

BIOMALLOXENE MODIFIED PROTEINS

By

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To Mom, Dad, Tim, Fred, Linda, Helen, Gail, Fred, and in
loving memory of Frank

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TABLE OF CONTENTS

page

ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	viii
ABBREVIATIONS.....	ix
SUMMARY.....	xi
CHAPTER I	
1. INTRODUCTION.....	1
Applications of Metalloenzymes in	
Biological Systems.....	1
Metalloenzymes as Biological Model Agents.....	2
Metalloenzymes as Medical and Immunological	
Imaging Agents.....	3
Metalloenzymes as Therapeutic Agents.....	4
Metalloenzymes as Biological Catalytic Agents.....	5
2. SINGLE-SITE MODIFICATION OF CYTOCHROME C.....	14
Background.....	14
Results and Discussion.....	21
Experimental Section.....	23
3. MULTI-SITE MODIFICATION OF BOVINE BROWN ALBUMIN AND	
KIDNEY LIVER HEMOGLOBIN.....	27
Introduction.....	27
Results and Discussion.....	34
Experimental Section.....	36
4. SYNTHESIS.....	50
Introduction.....	50
Alkylating Agents.....	101
Metalloenzyme Substrate.....	122
Experimental Section.....	123
REFERENCES.....	127
BIBLIOGRAPHICAL SECTION.....	127

CONT OF TABLE

PAGE

1.1	Biodistribution of Metalloporous Derivatives.....	4
2.1	Organic Nitroating Reagents and Their Organometallic Analogs.....	11
2.2	Chemical Shift Differences Between DMSO and Its Corresponding Sulfonium Ion Resonance.....	43
2.3	Relative Rate for the Reactions of Nitro with METHANOL.....	42
2.4	Relative Rate for the Reactions of Nitro with FORMIC ACID IN CDCl ₃ AT 50°C.....	43
4.1	¹³ C NMR Chemical Shifts of organic and Organometallic Compounds.....	88
4.2	¹³ C NMR Chemical Shifts of organic and Organometallic Compounds.....	90

LIST OF FIGURES

Page

2.1	Cytochrome c is an electron shuttle between membrane bound cytochrome c oxidase and cytochrome c reductase.....	17
2.2	The amino acid sequence of horse heart cytochrome c.....	18
2.3	The heme region of HbHc showing the coordination sphere of the heme iron.....	19
2.4	140 MHz ^1H NMR spectrum of 1.7 mM horse heart ferricytochrome c in D_2O at 25°C.....	21
2.5	The reactions of iodometric acid with several amino acid side chains.....	22
2.6	A typical ^1H NMR spectrum of an alkylating reagent (25,000 M/L) that has reacted with HbHc to produce the corresponding sulfonium ion.....	27
2.7	Reverse projection of the sulfonium sulfur methylene band showing the inequivalence of the methylene protons.....	28
2.8	Kinetic plot for the reaction of HbHc with N-methylmaleimide at 50°C using the integration of the sulfonium ion triplet (δ 3.48) vs time....	33
2.9	Plot of $\ln[A - x]$ versus time for the reaction of N-methylmaleimide with HbHc at 50°C (A = integral of the sulfonium methyl resonance at δ 3.48, x = integral of the methyl peak at infinite time). ..	38
2.10	Plot of the integral versus time for the methyl triplet (δ 3.48) of the sulfonium ion produced from the alkylation of HbHc by N-methylmaleimide at 50°C.....	40
2.11	The plot of $\ln[A - x]$ vs t for the reaction of N-methylmaleimide with HbHc using the sulfonium methyl triplet.....	43

3.1	General Mechanism of Carbodiimide Coupling.....	66
3.2	UV-Visible spectrum of native Bovine Serum Albumin (BSA) in 50 mM phosphate buffer, pH 7.0 (1 cm path length).....	74
3.3	Spectrum of Ferrocenylhistyric acid modified Bovine Serum Albumin in 50 mM phosphate buffer, pH 7.0 (1 cm path length).....	75
3.4	UV-Visible spectrum of Ferrocenylhistyric acid in 50 mM phosphate buffer, pH 7.0 (1 cm path length).....	76
3.5	UV-Visible spectrum of Native Baphos Linpet Memocyanin in distilled water (0.1mg/ml, 1 cm path length).....	80
3.6	UV-Visible spectrum of Ferrocenylhistyric acid Modified baphos Linpet Memocyanin (fraction 1) in 50 mM phosphate buffer, pH 7.0 (1 cm path length).....	81
3.7	UV-Visible spectrum of Ferrocenylhistyric acid Modified baphos Linpet memocyanin (fraction 2) in 50 mM phosphate buffer, pH 7.0 (1 cm path length).....	82
4.1	Monosubstituted ferrocene derivatives prepared and discussed in chapter 4.....	88
4.2	Room temperature ^1H NMR spectra of monosubstituted ferrocene derivatives.....	92
4.3	Room temperature ^{13}C NMR spectra of monosubstituted ferrocene derivatives.....	99
4.4	FTIR 1-6000 cm^{-1} spectra of derivatives prepared.	91
4.5	The FTIR peak sequence.....	92
4.6	Typical UV-Vis spectrum of a monosubstituted ferrocene derivative (FcMethCl).....	95
4.7	Typical UV-Visible spectrum for a monosubstituted ferrocene derivative (FcMethCl).....	96
4.8	Synthesis of FcMeCl.....	99
4.9	Synthesis of FcMethCl and FcMethCl.....	100

4.14 Newman projection of the methylase carbon of P ₀ ADH ₂ showing the enantioic inequivalence of the two methylase protons.....	117
4.15 The portion of the ¹ H NMR spectrum of P ₀ ADH ₂ that contains the doublet of triplets.....	118
4.16 CDH ₂ NMR spectrum of P ₀ ADH ₂	120
4.17 Synthesis of organic analogues.....	122
4.18 The synthesis of P ₀ ADH ₂	123
4.19 Room temperature ¹ H NMR spectrum of P ₀ ADH ₂ in CD ₃ OH (referenced to CD ₃ COO at δ 3.35).....	124
4.20 ¹ H NMR spectra of the phosphorylase derivatives prepared.....	127
4.21 Synthesis of the metalloenzyme ketocoids.....	129
4.22 Reaction schemes for the synthesis of Opfcp8(H ₂ C(O)CH ₂ OH).....	130

ABBREVIATIONS

BGA	bovine serum albumin
CP	cyclopentadienide
Cyt c	cytochrome c
DM	1-[2-dimethylaminopropyl]-2-ethylcarbodiimide
DAP	2,2-dimethyl-2-aziridinecarboxylic acid
FA	oxidized flavin adenine dinucleotide
FAH ₂	reduced flavin adenine dinucleotide
Fe	ferrocene
FeAACl	chloroacetyl ferrocene
FeAG	acetoacetyl ferrocene
FeAAcAc	acetoacetyl acetyl ferrocene
FeAAcC	acetoacetyl acetyl ferrocene
FeAAc	ferrocenylacetic acid
FeCOOH	ferrocenylacetic acid
FePhenAAc	p-ferrocenylacetic acid
FePhenC	ferrocenylacetic acid
FE-IR	Farmer infrared infrared spectroscopy
HbC	horse heart cytochrome c
HbC(II)	horse heart ferrocyanochrome c
HbC(III)	horse heart ferricytochrome c
KL	Knight ligand complex
KfPhenAAc	p-ferrocenylacetic acid
KfPhenAAcC	p-ferrocenylacetic acid
KfPhenAAcC	p-ferrocenylacetic acid
Kf	nuclear magnetic resonance
Kf	ferrocene
KfAAc	ferrocenylacetic acid

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NEUTRALIZED MODIFIED PROTEINS

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Several ferrrocene derivatives have been prepared for the purpose of modifying the proteins bovine serum albumin (BSA), legume hemophorin (HF), and horse heart cytochrome c (CCc). New ferrocenyl alkylating agents containing a functional group (haloacetyl or haloacetamide) either directly bound to one of the ferrocenyl cyclopentadienyl ligands ($\text{CpFeCp}(\text{CH}_2\text{CH}_2\text{X})_1, \text{CH}_2\text{CH}_2\text{N}$, where $n=0,1$ and $\text{X}=\text{Br}$ or I) or in the para position of phenylferrrocene ($\text{CpFeCpC}_6\text{H}_4\text{N}(\text{CH}_2\text{CH}_2\text{X})$) have been prepared and characterized. Each ferrrocene derivative has an argonide anion that has been used to modify the methionine residues in cytochrome c. The long-term goal of the project is to selectively modify the methionine 48 residue in horse heart cytochrome c with a ferrrocene derivative and to study conformational changes in HCCc by studying the rates of

electron transfer between the iron heme of the cytochrome and the iron center of the metalloenzyme. The preparations and characterizations of the ferrocenyl derivatives are presented. In addition, relevant model studies for reaction of the ferrocene derivative with the proteins have been completed. Alkylation rates of the model compound by ferrocene derivatives and their organic analogues are compared. Methods of protein modification are described, together with methods for verifying both the extent and site of modification. The technique of quantifying micromolar protein concentrations by using inductively coupled plasma-atomic emission spectrometry (ICP-AES) of sulfur in the vanadate-V is presented.

A second series of metalloenzyme derivatives was prepared for immunological studies. Ferrocenyl and ruthenocenyl ketoxides $\text{Cp}^*\text{CpCp}(\text{O})(\text{CO}_2)_2\text{COOR}$ where R-Ha or Fe and n=0,1) were prepared and coupled to two carrier proteins, keyhole limpet haemocyanin and bovine serum albumin, for the purpose of developing antibodies against the metalloenzyme. Preparations and characterizations of the metalloenzyme ketoxides and the methods of multi-site protein modification and quantification are described. Results from hybridoma laboratory studies of these ferrocenyl antigens are presented. Potential uses of metalloenzyme-specific antibodies as radiotracers and synthetic enzymes are discussed.

CHAPTER 1 INTRODUCTION

Applications of Metalloenes in Biological Systems

Since Ruedy and Pauson first prepared ferrocene in 1950, organometallic chemists have concentrated their efforts on synthesis and reaction mechanisms of new organometallic compounds [1]. The original metallocene compound, ferrocene, contains a low spin d^6 iron(II) center bound to two cyclopentadienyl rings (Fig) in a sandwich-type structure. Ferrocene is an orange, air-stable compound that obeys the 18 electron rule. Only a few groups have explored the potential biological applications of organometallic compounds. A general overview of applications of metallocenes in biology, medicine, and toxicology has been published by Zaslavski and co-workers [2].

Chapter 1 will introduce several applications of metallocenes, which can be grouped into four categories (use of metallocenes as relax agents, therapeutic agents, imaging agents, and catalytic agents) and will give a brief overview of our applications of metallocenes with respect to the categories listed above. In chapter 2, the ferrocene

derivatives that have been investigated as potential single-site modifying agents for development as biological redox probes are discussed. Use of metalloocene derivatives as ligands for the production of antibodies against the metallocoenes is discussed in chapter 3, together with the potential applications of the metallocoene-specific antibodies for development of immunoassays and synthetic enzymes. Preparation and characterization of the metallocoenes and organic reagents used for both protein modification projects are presented in chapter 4. Chapter 5 summarizes the results of this study.

Metallocoenes as Biological Redox Agents

As of December 1987, Chemical Abstracts listed 203 publications on a variety of ferrocene modified electrodes. A good review of recent research in ferrocene modified electrodes is provided by E. A. G. Hill [1]. One of the most interesting uses of a ferrocene derivative in bioelectrochemistry has been in the development of a glucose sensor, which can directly assay the glucose concentration in whole blood [2]. In animals, glucose is broken down by glucose oxidase to gluconolactone. Glucose oxidase, a structurally rigid ribonucleoprotein (MW 100 kDa) with a radius of 40 Å, consists of two identical polypeptide chains, each containing a FAD/FMN₂ (oxidized and reduced

liverin edeine dimethylamide) centers. The FAD/FADH₂ centers are buried deep within the protein. When glucose diffuses into the enzyme, the FAD center is reduced to FADH₂ via transfer of two electrons from the glucose, and, consequently, the glucose is oxidized to gluconolactone. Since electron transfer rates decay exponentially with distance [2], the large radius of the enzyme prevents communication between the FADH₂ centers buried deep in the glycoprotein and simple metal or semi-metal electrode surfaces at a practical rate. Consequently, direct electrochemical monitoring of glucose oxidase in the presence of varying levels of glucose shows no variation in the cyclic voltammogram obtained [3]. The conventional method of determining glucose levels in the blood required dilution of the blood with argon-saturated buffer and introduction of an appropriate electro and chemically reactive species, such as oxygen, to produce a species, like H₂O₂, that can be studied [4]. Goss, Hill, and co-workers have demonstrated that ferrocenedimethanesulfonic acid and ferrocene carboxylate are effective electron shuttles for the glucose oxidase system [5]. Recently, Negele and Heller have reported the development of a ferrocene-modified glucose oxidase electrode, which can be used to directly monitor levels in whole blood [6]. The electrode was prepared by coupling ferrocenedimethanesulfonic acid to the lysine amino acid side chains of glucose oxidase by using

the water-soluble carbodiimide coupling reagent, 1-[3-diethylaminopropyl]-3-ethylcarbodiimide hydrochloride (EDC), under slightly deaerating conditions (3 M urea) [8]. The ferrocene groups provide a conductive pathway between the FAD/FAD_{ox} centers buried deep within the glucose oxidase and the working electrode, thus allowing the direct determination of glucose levels in whole blood.

Ferrocene derivatives have also been used as metalloprotein redox titrants. In order to understand the more complex biological systems involving the transfer of an electron between two redox proteins, model studies involving electron transfer between small inorganic complexes and a redox protein have been pursued by several groups [7-10]. Ferrocene has hydrophobic Cp rings and a π -system that delocalizes the iron electron density, and, as a neutral molecule, ferrocene can probe hydrophobic areas of proteins that often contain the active site. Ferrocene derivatives as metalloprotein redox titrants have additional advantages over simple inorganic complexes, including high intrinsic electron transfer ability, conformity to Marcus theory for outer sphere electron transfer, inertness to substitution in both oxidation states, and a wide range of redox potentials available through derivatization [11]. The high intrinsic electron transfer ability, demonstrated by the rather large self-exchange rate constants of ferrocene derivatives ($10^6 - 10^8 \text{ M}^{-1} \text{ s}^{-1}$) [12], indicates that the ferrocene system is

family is electron transfer and has no large reorganizational barriers, that would prevent ferrocene derivatives from being good redox titriments. The series of ferrocene derivatives studied by Carnay, Flaudouat, and coworkers conform to Marcus theory over a significant range of thermodynamic driving forces ($\Delta E^\circ = -0.23$ to $+0.31$ V) and represent the first extensive series of closely related complexes to behave as predictably over such a large thermodynamic range [11]. The wide range of redox potentials available in ferrocene derivatives makes them well-suited as metalloprotein titriments for a variety of redox proteins including iron-sulfur proteins [12], cytochromes c [13], and copper proteins [14].

Metalloporphyrins as Medical and Technological Titrimetric Agents

Metalloporphyrins containing the proper quasi-sticking metal center can be used as radiotitrants. Specifically, ferrocene derivatives containing an ^{59}Fe nucleus [15] and ruthenocene derivatives [16] containing a ^{96}Ru or ^{101}Ru nucleus have potential applications as radiotitrants. Of the three metal centers mentioned above, ^{96}Ru has the best quasi-sticking and clinical properties [17], which include a minimal half-life ($t_{1/2} = 2.6$ days) good metal-chelation graphs with resulting power (energy < 500 keV), ^{96}Ru has quasi energy of 215-328 keV, and stability in vivo. ^{101}Ru ruthenocene

derivatives are easily prepared via Cp transfer by heating $^{99m}\text{TcCl}_4$ and the corresponding ferrocene derivative [48]. Taylor, Mansel, and others have prepared a series of ^{99m}Tc and ^{51}Cr ruthenocene derivatives that show organ specificity in their biodistribution [18-22]. The biodistribution of ruthenocene derivatives largely depends on the Cp substituent. Mode of administration and size of the subject also play small roles in the biodistribution of the ruthenocene derivative (see Table 1.1).

Table 1.1. Biodistribution of Metallocene Derivatives.

Metallocene Derivative	Admin. Route	Primary Organ Target
Azetyl	PO, IV	adrenals, kidneys
Carboxylate	IP	kidneys
Carboxylaldehyde	IP	kidneys
Cinnamyl-9a	IP	liver, lungs, spleen
Cinnamyl-9b	IP	thymus
Ferrocene	ID	lungs
Ethylmethyl	IP	adrenals, kidneys
Ethylene	IV	lungs

Abbreviations used: PO, peroral; IP, intraperitoneal; ID, inhalation; IV, orally.

Metallocenes have also been useful in histochemical and immunochemical analyses. Commonly, antibodies are labelled

either with an iron-containing protein, ferritin [21], or peroxidase [22] for electron microscopy experiments in order to visualize surface cellular antigens/determinants.

Stuhler et al., has conjugated a ferrucene derivative with a 40 kDa lectin for the indirect identification of lectin receptors by using electron microscopy [23]. The advantage of the ferrucene method is that ferrucene, a small molecule, can pass through the cell membrane, whereas the other two methods require that the cell be treated with disrupting agents or undergo enzymatic digestion of membrane proteins to create a hole through which the compound may enter. Unfortunately, the latter methods lead to distortion of the cell shape.

Tiggesmann and Geyrhofer have labeled the Fab fragment of an antibody with ferrucenylsuccinylsuccinic acid to enable the identification of intracellular structures via electron microscopy [2]. The ferrucene method is considered to be superior to other labelling procedures because the coupling process does not influence the binding capacity of the conjugate and because the researchers were able to identify the intracellular antigens without background effects due to destruction of the cellular ultrastructure [2]. Gade and co-workers have developed a method using ferrucene derivatives for quantifying both antigens and antibodies via the general precipitation method followed by atomic absorption [24].

Metalloenes as Therapeutic Agents

Medical applications of metalloenes include use as anti-cancer agents. Early work by Rosenberg et al. showed that the surfaces of tumor cells have carboxylic acids while normal cells lack such sites [26]. Florin et al. have prepared a series of polynuclear ferrocene derivatives designed to bind to the carboxylic acid sites of the tumor cells [26]. While the polynuclear ferrocene derivatives showed no anti-tumor activity, the precursor compounds prepared did show low but significant anti-tumor activity. Florin found that upon binding of more than 10 ferrocene polynuclear derivatives per Ehrlich ascites tumor cell, the tumor was eliminated from the body [26].

Impressive anti-tumor activity is displayed by metalloene dihalides and pseudo-dihalides. Because the metalloene dihalides structurally resemble the currently used anti-cancer agent cisplatin [27-28] a variety of metalloene and metalloene dihalides have been investigated as potential anti-tumor agents. Like cisplatin, the metalloene dihalides have two leaving halides and are believed to act on DNA via the intramolecular and intermolecular mechanisms [29]. The metalloene derivatives and cisplatin are also similar in that each has a soft acidic center and two strongly electron donating ligands. The two most promising of these metalloene anti-cancer reagents are

titanocene dichloride and zirconocene dichloride, which have cure rates of 88-98% for a single intraperitoneal injection 24 hours after tumor transplantation and an increase in mean survival time for the treated animal of 80%.

General trends for the effectiveness of metallocene dichlorides as anti-tumor agents include a decrease in activity in a series (d^0 , d^2 , d^4) with increasing atomic weight and an optimum activity leading to 100 % cure rates obtained when the central metal is Ti, V, Nb, or Mo. [2]. Modification of the cyclopentadienyl rings of titanocene-dichloride diminishes the anti-cancer effectiveness in direct proportion to the number of substituents added [16].

Metallocenes as Biological Catalytic Agents

Recent interest in the development of synthetic enzymes led to the development of a synthetic enzyme with a metallocene catalytic site. Swalen's group has modified the β -cyclopentadiene cavity with the p-nitrophenylisobar of zirconocene acrylate such as that the ferrocene fits into the cavity while the zirconium side chain brings the ester near the catalytic hydroxyl group, thereby yielding a rate enhancement on the order of 10^3 [17]. When the acrylate side chain is incorporated into a fused ring system so that the ester is more rigidly held near the hydroxyl group, a rate increase by a factor of 3.3×10^3 is observed [18]. Upon

adding one more degree of steric constraint to the ferrocenyl system by attaching the ester to the fused ring system through a double bond, a rate increase of 1.9×10^4 was observed for the hydrolysis reaction relative to the unsubstituted reaction [31]. The rate of hydrolysis for the same system can be further enhanced by running the reaction in aqueous dimethylsulfoxide rather than water, producing an increase of 1.5×10^3 over the non-substituted system. A rate increase of 1.9×10^4 is well within the range of acceleration produced by enzymes. Breslow's work represents the largest acceleration of a reaction by a synthetic enzyme demonstrated thus far [31]. In addition, the rate of hydrolysis is also dependent on the enantiomer of the ferrocene derivative chosen [31]. In the case of 1-(carboxymethylene)-1,1'-ferrocenecyclopentene-p-nitro-phenylamide, the E enantiomer increases the rate of hydrolysis by 9.5×10^3 , while the Z enantiomer increases the rate by only 9.5×10^2 [31]. The enantiomeric preference of the E form over the Z form of the ferrocene derivative by a factor of 43 is the largest enantiomeric selectivity demonstrated for a hydrolysis reaction [31]. In addition, the ferrocenyl synthetic enzyme shows the largest enantiomeric selectivity demonstrated for a hydrolysis reaction.

Introduction to Ferrocenyl Work

The research herein involves the application of metallacycles as single-site modifying agents for the development of biological structural probes (chapter 1) and as multi-site modifying agents for the development of antibodies against metallacycles that may be used for the development of synthetic enzymes or biocatalysts (chapter 2). The single-site projects involved the development of a series of new ferrocenyl silylating reagents of the general types $\text{Cp}^*\text{Fe}(\text{Cp})(\text{CH}_2)_n\text{P}(\text{R})\text{C}(\text{O})\text{CH}_2\text{X}$ (where $n=0$ or 1 referred to as *ferrocenyl*, 1), $\text{Cp}^*\text{Fe}(\text{Cp})(\text{C}(\text{O})\text{CH}_2\text{X})_2$ (*ferrocenyl*), and $p\text{-Cp}^*\text{Fe}(\text{Cp})(\text{CH}_2)_n\text{C}(\text{O})\text{C}(\text{O})\text{CH}_2\text{X}$ (*ferrocenyl*). These reagents were prepared and investigated in model studies with ethylmethylsulfide to determine their ability to silylate the methionine 48 amino acid side chain in the model protein, cytochrome c (Chapter 3). The long-term goal of the project is to develop a ferrocenyl biological probe by incorporating a single ferrocene derivative into the cytochrome system. The metal center could also be used as a structural probe of conformational changes associated with changes in the protein's environment.

The first two general types of ferrocene derivatives prepared showed no reaction with E1788 or the protein under a variety of reaction conditions (see chapter 1) , whereas the last ferrocenyl compound did silylate the model

described. Comparisons between the organic and organometallic analogues are presented in chapter 3.

Single-site modified cytochrome c has been the subject of a considerable number of intramolecular electron transfer studies. Recently, Gray, Tiedj, and others have modified His 13 of cytochrome c with ruthenium complexes of the general type $Ru(SO_3)_2(L)(N_3)_2^{2+}$ (where L represents the ligands SO_3 or isocyanatimide) [34-36]. Work by Tiedj and co-workers, discussed in more detail in chapter 3, has presented the possibility that electron transfer in this system may be gated [35]. Further intramolecular electron transfer studies are needed to understand this interesting observation.

The potential advantage of a ferrocenyl modified cytochrome c system with respect to Tiedj's system is that the cytochrome c will be modified in a different area of the protein. The area we targeted is near the heme edge and has been implicated in the orientation of the redox protein toward its biological redox partners [36-38], whereas the His 13 site modified by Gray, Tiedj, and co-workers is located on the backside of the protein away from the heme edge. In addition to attempting to alkylate cytochrome c, ferrocenyl-carboxylic acid was used to modify cytochrome c by using the carbodiimide coupling method, which is described in the earlier section on ferrocene modified

electrodes and in more detail in chapter 3. Results of the electrochromic work are discussed in chapter 2.

In a second project discussed in chapter 3, a metalloocene derivative has been used as a haptan for the development of metalloocene-specific antibodies. Since ferrocene has been shown to elicit a strong immune response, we have modified the carrier proteins, bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), with a series of ferrocenyl ketonamides of the general type $\text{CF}_3\text{CH}_2\text{C}(=\text{O})[(\text{CH}_2)_m\text{C}(=\text{O})]_n\text{OH}$ (where $m=2,4$ and $n=0,1$). By using the carbodiimide coupling method, we have conjugated ferrocenylbutyric acids to both BSA (in Fe_2/BSA) and KLH (1:1 Fe_2/KLH subunit). Initial immunological reports from our workers indicate that metalloocene-specific antibodies are not being produced against the ferrocene derivative. Our long-term goal for the metalloocene antibodies project is to develop synthetic redox-active metalloproteins by interspersing the metalloocene into the antibody. Results of the metalloocene antibody project are presented in chapter 3. An overall summary is presented in chapter 5.

CHAPTER 2
SINGLE-SITE MODIFICATION OF CYTOCHROME C

Background

The long-term goal has been to modify a single amino acid side chain on a protein with a transcription metal complex. The modified protein will be used in electron transfer studies to investigate conformational changes associated with changes in the protein's environment (such as changes in pH, ionic strength, and temperature). Conformational changes in proteins are important in many biological processes. For example, the simplest components of the complex cellular regulatory system are the quaternary proteins (allosteric proteins). The interaction of the binding sites for the various metabolites, mediated by the quaternary structure of the allosteric proteins, accounts for the regulatory properties of the proteins. Specifically, myoglobin, an oxygen-binding allosteric protein, contains a single binding site for a metabolite (O_2). Allosteric proteins are controlled by a second set of metabolites, allosteric effectors, that bind to separate sites on the quaternary protein. An allosteric effector does not act as a switch that turns the activity of the protein "on" or "off," but is analogous to a light dimmer that regulates brightness and requires little energy.

Postnikova and co-workers have modified spin labels to include a functional group capable of covalently attaching the spin label to the surface of cytochrome c, thus incorporating a stable nitroxyl radical into the protein [38]. Methods similar to those employed by Postnikova could be used to covalently bind a metal containing moiety onto the surface of cytochrome c. Chapter 2 outlines the choice of protein, metal containing moiety, functional group for covalent attachment, amino acid target, method of site-specific modification, and method for determining both the extent and site of modification for the single-site modification project. In addition, the results of metal studies and attempts at protein modification are discussed.

Choice of Protein

The simple redox protein, cytochrome c, obtained from horse heart (HHC) was chosen as the initial protein for the single-site modification project. HHC has been used in many electron transfer studies because it is readily available, stable to application of standard biochemical techniques, and inexpensive. The biological function of cytochrome c is to shuttle a single electron between oxidized-bound cytochrome c oxidase and cytochrome c reductase of the oxidative phosphorylation assembly, which is in the mitochondria of eucaryotic cells (see Figure 2.1).

Heme is a relatively small protein with a molecular weight of 18000 Da. Horse heart cytochrome c is composed of a single polypeptide chain of 104 amino acid residues (see Figure 1.8) and an iron containing heme group. The heme is located in a hydrophobic channel with one edge of the heme exposed to the solvent. The porphyrin is covalently bound to the protein by two cysteine residues. The heme iron's fifth and sixth coordination sites are occupied by a sulfur atom from methionine 80 and a nitrogen atom from histidine 18 (see Figure 1.10). X-ray analyses of ferricytochrome and ferriocytochrome show little or no difference in the Fe-hist 18 bond length [40], although chemical studies indicate that the Fe^{3+} -hist 18 bond is not very stable with respect to ligand displacement of the thioether sulfur atom [41]. Simple ligands, such as cyanide, azide, and iodide can easily displace the hist 18 donor atom of heme(III) [42]. Heme has an isoelectric point near pH 10 and has many amino acid residues that are conserved throughout a wide range of species [43]. The residues that are conserved are mostly residues located near the heme pocket [43]. Cytochrome c has a high lipase content and no disulfide bonds. The reduction potential of the heme $Fe^{3+/2+}$ at neutral pH is 0.160 V vs NHE. The proposed mechanism for electron transfer between HemeC and its redox partners is through space from the heme iron in cytochrome c to the heme of the corresponding redox partner [44]. The surface of cytochrome

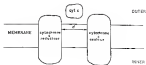


Figure 2.1. Cytochrome *c* is an electron shuttle between membrane bound cytochromes *c* oxidase and cytochromes *a* and *a₃*.

Acetyl-4 10 20 30 40 50
 R F E E G E E I F Y Q E C A Q C E Y Y E E G G E E E
 60 70 80 90 100
 T G P H L E E L F G E E T G G A F G P Y Y T G A E E E E E
 110 120 130 140 150
 I T E E E E T L E E Y L E E P E E Y I F G T E E I F A G E
 160 170 180
 E E E T E E E L I A T L E E A T H D-COOH

Figure 3.3. The amino acid sequence of horse heart cytochrome c.



Figure 2.3. The base region of KCCO showing the coordination sphere of the base iron.

α near the exposed base edge is composed of a large number of lysine amino acid residues (side chain = $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$). At physiological pH, the lysine side chains are protonated creating a patch of positive charge. The positive patch has been implicated by several groups as an orienting factor for the protein toward the ribonuclease (38-40).

Cytochrome c has been extensively characterized by many analytical techniques including X-ray crystallography, nuclear magnetic resonance (NMR), postcysteic acidification, high pressure liquid chromatography (HPLC), and UV-visible spectrophotometry. An X-ray crystal structure of hRCC(II) has been determined by R. E. Dickerson and co-workers to 1.9 Å [43]. The corresponding hRCC(III) crystal structure has been elucidated to 1.8 Å [44]. The structural differences between the two forms of the protein are minimal. The major structural changes are in the region around the His 63 and Tyr 74 residues [44].

Williams and coworkers have published many papers on the ^1H NMR spectra of hRCC(II) and hRCC(III) and assigned the majority of the aliphatic and aromatic resonances (see Figure 2.4) (45-49). Most important to this study are the three sharp singlets in the ^1H NMR spectrum of hRCC(II) that appear at δ 2.12, 2.00, and -3.18 corresponding to the methyl resonances of Met 45, the N-acetyl tarsule of the protein, and Met 48, respectively. The Met 45 and N-acetyl methyl resonances were assigned via an alkylation experiment



Figure 2.4. 100 MHz ^1H NMR spectrum of 0.7 mM human heart ferritythionin in D_2O at 25°C (referenced to external TMS).

[45]. Selenosulfonic acid was used to alkylate the Met 45 residue of HbCo(II) producing a sulfonium center. The methyl resonance of the alkylated Met 45 is δ 0.79 downfield from the original Met 45 methyl resonance [46]. The Met 45 methyl resonance appears at δ -30 in the ^1H NMR spectrum of HbCo(III) and at δ -31.28 in the ^1H NMR spectrum of HbCo(II) [46]. The alpha and beta methylene protons of the Met 45 residue located at δ -0.87 and -0.10 are very sensitive to changes in the heme environment [46]. Many ^{13}C NMR resonances of the spectra of HbCo have been assigned by R. Suck and co-workers [48].

Cytochrome c has been cleaved by trypsin and other proteolytic enzymes into known oligopeptide fragments. Trypsin digestion has been a helpful technique in establishing which fragment of HbCo has been modified. Once the modified protein has been digested, the fragments are separated by HPLC chromatography. The chromatographed fragment with different elution properties from the native cytochrome c fragments can be identified as the modified amino acid residue. Gray, Tided, and co-workers have used this technique to determine which of the cytochrome c peptide fragments contained the amino acid modified by $\text{Fe}(\text{NH}_4)_2(\text{S}_2\text{O}_8)^{2-}$ [34]. Once the fragment containing the bound ruthenium complex was established, the specific amino acid binding site was determined by ^1H NMR experiments [34].

The electronic absorption spectra of HbOs contain d-d bands and charge-transfer bands [54]. HbOs(II) has an electronic absorption spectrum containing absorptions at 430 nm, while HbOs(III) does not absorb at this wavelength [54]. The electronic spectrum of HbOs(III) contains an absorption band at 438 nm, which the spectrum of HbOs(II) lacks [52]. Oxidation of the iron center in HbOs(II) is typically achieved by using either $\text{Ce}(\text{Phen})_3^{IV}$ or ferricyanide, while reduction of the heme iron is easily accomplished with sodium dithionite or ascorbic acid. If a ligand displaces the distal sulfur from the HbOs(III) iron center, the electronic absorption spectrum of the protein will lack a characteristic absorption band at 438 nm [41].

In order to understand the more complex biological electron transfer reactions between cytochromes c and its redox partners, several groups have investigated the electron transfer reactions between simple model compounds and cytochromes c [3-18]. The inorganic complexes serve as models for the cytochromes c oxidase and reductase redox partners of cytochromes c, which are not routinely available in pure form.

One of the most studied inorganic electron transfer complexes is ferricyanide, which reacts with ferrocyanochrome with an overall rate constant between 4×10^6 and $58 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [7]. In an elegant set of chemical modification studies, Shown and Miller determined that when any one of

the lysines surrounding the exposed heme edge are chemically modified, the rate of electron transfer between the ferrioxalate and cytochrome *c* was significantly reduced [24]. Modification of a lysine side chain not located near the heme had no effect on the rate of electron transfer [24]. Electron transfer rates of simple inorganic complexes, such as $\text{Co}(\text{Phen})_3^{3+}$, $\text{Ru}(\text{NH}_3)_6^{3+}$, $\text{Fe}(\text{EDTA})^{2+}$, and $\text{Fe}(\text{CN})_6^{3-}$, correlate well with rates expected from Marcus theory of outer-sphere electron transfer, and these results suggest that all the electron transfer reactions occur by a common mechanism [11]. Furthermore, single-site modification of the lysine side chains near the cytochrome *c* heme edge have been shown by Ferguson-Miller and co-workers to decrease the reactivity of cytochrome *c* with cytochrome *c* oxidase, thereby implicating the heme edge as the site where in vivo electron transfer may take place [25]. Similar results were obtained for the interaction of modified cytochrome *c* and succinate-cytochrome *c* reductase [26].

From the interaction of cytochrome *c* with inorganic complexes, one can see that those complexes that can effectively access the heme edge are more efficient reagents than complexes that are excluded from close approach to the heme [11]. Specifically, small complexes with hydrophobic ligands and metal to ligand π -bonding (e.g., $\text{Co}(\text{Phen})_3^{3+}$) are more facile at electron transfer than complexes with hydrophilic ligands and metal-ligand bonding

that does not allow for delocalization of the metal electrons onto the ligands (i.e. $\text{Ru}(\text{NH}_3)_6^{3+}$) [11].

Ferrocene has the advantages of the $\text{Co}(\text{phen})_3^{3+}$ system since ferrocene can delocalize the iron electron density through the Cp ligands and is hydrophobic. Ferrocene, a neutral molecule, may have greater access to hydrophobic areas than charged molecules. In addition, ferrocene derivatives have high intrinsic electron transfer ability as evidenced by their high self-exchange rates [12].

Derivatives of ferrocene are inert to substitution in both oxidation states, thus there are no large reorganizational barriers to electron transfer arising from bond breaking or bond making. Ferrocene derivatives have a variety of redox potentials available through derivatization of the Cp ring.

Based on the observations about ferrocene above, Shiner, Fladman, and others have prepared a series of ferrocene derivatives, which have been used as metalloprotein redox titrants for cytochrome c [13], blue copper proteins [14], and iron-sulfur proteins [15]. The ferrocene derivatives used in the intermolecular electron transfer studies have redox potentials ranging from $E^\circ = 0.25$ to $+0.31$ V [11]. The Fladman group suggested that ferrocene derivatives were excellent metalloprotein redox titrants because their electron-transfer rate constants were comparable to rate constants obtained for the hydroquinone, reduced isopyronic complexes above and much greater than

the rate constants determined for the sigma-bonded, hydrophilic complexes. Temperature dependence studies [11] revealed that the interaction between the protein and the ferrocene derivatives is a single bimolecular collision, suggesting that minimal protein structure reorganization is needed for electron transfer with the ferrocene derivatives ($\Delta S^\ddagger = 4-8$ kcal/mole, $-\Delta H^\ddagger = 0-13$ e.u.). Garvey, Fiedorowicz, and co-workers suggest that the unsubstituted Cp ring may penetrate the hydrophobic channel containing the heme prior to the electron transfer between the two iron sites [11]. The ferrocene derivatives studied represent the first extensive series of closely related complexes to behave as predicted with respect to Marcus theory over such a large thermodynamic range [11].

While intermolecular electron transfer studies provide us with considerable information, intramolecular electron transfer studies offer the following advantages: a fixed protonic site at a known location, the ability to vary the protonic site by varying the functional group needed for covalent attachment, and the ability to vary the driving force for the electron transfer reactions by varying the substituents on the metal complex.

Recently, single-site modified cytochromes c have been the subject of a number of intramolecular electron transfer studies [13, 14, 25]. Grey, Isied, and co-workers have modified the imidazole side chain of His 33 by ruthenium

amine complexes of the general type $\text{Ru}(\text{NH}_3)_5(\text{Ame})\text{Cl}_2$ (where Ame represents either NH_3 or isocyanotrimethyl) [34, 35]. The ruthenium complex reacts with the isideazole to displace the water ligand with the nitrogen atom of the isideazole (see scheme 1a) [36].



The rate constant for intramolecular electron transfer between the Ru(II) center and the Fe(III) heme center in the ruthenium modified cytochrome c is 33 s^{-1} , which is on the same order of magnitude as rate constants obtained for a number of conformational changes associated with the protein [34]. Takushi et al. have observed the presence of intermediates formed during the reduction of $\text{RuO}(\text{II})$ but not during the re-oxidation and have suggested that the reduction pathway is different from the oxidation pathway [32]. Furthermore, x-ray studies show conformational differences between the oxidized and reduced forms of cytochrome c [32-35], which are not apparent by simply comparing the crystal structures of $\text{RuO}(\text{II})$ and $\text{RuO}(\text{III})$ [40, 41]. These results raise the question of whether electron transfer or a protein conformational change is the rate limiting step for electron transfer in cytochrome c [35]. To investigate the rate limiting step in the electron transfer reaction, Isied's group prepared a similar

ruthenium complex, by substituting an isocyanideamide ligand for one of the amine ligands [35]. The new ruthenium complex was also bound to the His 13 residue of cytochrome c. The driving force for electron transfer in the new ruthenium-cytochrome c system is nearly the same as the driving force in the initial ruthenium modified cytochrome c, but in the opposite direction [35]. Surprisingly, the rate of intramolecular electron transfer from the Fe(II) heme center to the Ru(III) center of the isocyanide complex was much slower ($>10^5$ times slower) than the rate of electron transfer for the corresponding rutheniumammine complex [35] and leads led to conclude that electron transfer in cytochrome c is directional, with the protein envelope presumably involved in the directionality [35]. Further intramolecular electron studies are needed to understand this interesting observation.

Choice of Ferricenium Coupling

We have chosen to use ferrocene as our redox containing complex, since ferrocene is an excellent metalloprotein redox titrant. In addition, the synthetic chemistry of ferrocene has been well established and its derivatives are usually air-stable. The reduction potential of a ferrocene derivative can be altered by changing the substituents on the Cp ligand, thus avoiding changes in the

primary coordination sphere of the iron center. A series of new ferrocene derivatives have been prepared for this study. The synthesis and characterization of these compounds are presented in detail in chapter 4.

Choice of Amino Acid Target

Interest lies in modifying a single amino acid site of cytochrome c close to the region where biological electron transfer takes place (the exposed heme edge). As mentioned before, the region surrounding the heme is largely composed of a number of lysine residues and a single methionine residue. By choosing a relatively infrequent amino acid for modification, the chances of modifying only a single amino acid site are increased. Methionine, tyrosine, and tryptophan are infrequent amino acid residues. Of the three residues, methionine is located in the region of interest. In addition, there are established methods for exclusively modifying the methionine 48 residue. The Met 48 residue has been selectively modified by Williams for their 1988 alkylation studies described earlier in this chapter [48]. Based on Williams' results, selective modification of the methionine 48 residue with a ferrocenyl alkylating reagent appeared feasible.

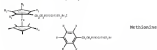
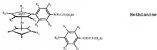
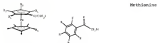
Shaw has published a general review on site-specific reagents that have been used to modify two proteins,

alanyltypsin and trypsin, and lists a number of organic alkylating reagents that have been used to modify methionine (see Table 3.1) [34]. All of the methionine alkylating reagents listed have a benzene ring and a haloacetyl (or haloacetamide group) in common. Based on the benzoyl Cp ring's ability to undergo electrophilic substitution reactions that the benzene ring can also undergo, ferrocene is considered to be the organometallic analog of benzene [32-35]. Consequently, several organometallic haloacetyl and haloacetamide compounds and several of their organic analogues have been prepared for our alkylation experiments (see Table 3.1). Haloacetyl and haloacetamide derivatives, similar to the new ferrocenyl alkylating reagents, show remarkable versatility for selectively alkylating amino acid side chains (see Figure 3.8).

Bovine heart cytochrome c contains 1 cysteine, 1 methionine, 10 lysine, and 4 histidine residues that are potential sites for alkylation [36]. When the pH is kept below 5.5, the Cys, Lys and His side chains are protonated and, therefore, much less reactive toward alkylation [37]. At pH 5.5 the methionine residues are the most reactive residues available in cytochrome c for alkylation. The Met 68 residues can be selectively modified over the Met 69 by alkylating the protein in its reduced form [38]. By reducing the heme iron a more stable bond is formed between

Table 2-3.
Organic Alkylating Reagents and Their Organometallic
Analogues.

Organic Reagent	Organic Reagent	Site of Modification ^a
-----------------	-----------------	-----------------------------------



^a Site of modification is cholecysteinyl (48).

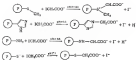


Figure 3.8. The reactions of glutathione acid with several amino acid side chains.

the heme iron and the sulfur donor atom of Met 88, thus decreasing the nucleophilicity of the Met 88 site.

The ferrocenyl alkylating reagents prepared are of two basic types: one where the haloacetyl (or haloacetoxy) functional group is directly attached to the Cp ring and one where the functional group is para to the ferrocenyl moiety across a benzene ring. Iodoacetyl-ferrocene (Fukaki), which has been prepared, is an organometallic analog of phenacylbromide, which was used by Schramm and Lannon [48] to modify methionine residues in chymotrypsin. Iodo and bromoacetylhaloethylferrocene (Fukaki, 8c) were prepared as the organometallic analogues of S-(β -phenethyl)- α -bromoacetoamide, which was also used by Schramm and Lannon [48] to modify methionine residues in chymotrypsin. p-bromoacetylhalophenylferrocene was prepared as the electrocyclic organometallic analog of p-bromo-acetamide, which has also been used as a methionine modifying agent [49] by Schramm and Lannon.

Methods for Determining the Extent and Site of Modification

The following analytical methods were developed to determine the extent and site of protein modification by the ferrocenyl reagents. Electrode absorption spectra were run on the pooled ion-exchange fractions of potentially modified cytochromes c to estimate the protein concentration

and detect any changes that might be associated with modification of the protein. The protein solutions were washed with deionized water to remove the DTT and diluted to give samples of protein with a base line concentration of about 1 ppm, as determined by using UV-visible spectrometry, for ICP-AES (inductively coupled plasma atomic emission spectrometry) analysis.

We have developed an ICP-AES method for the accurate determination of elemental concentrations of proteins with known sulfur contents. A complete description of our method is given elsewhere [20], but the method is briefly described here. Temperatures in the ICP-AES plasma torch are sufficiently high to excite very non-metallic elements [20], such as sulfur, which has an emission line at 180.73 nm and a detection limit of 15 $\mu\text{g/L}$ [21]. Proteins with known contents of the sulfur-bearing residues, cysteine and methionine, can then be quantified at the picogram level by ICP-AES provided the instrument is equipped with evaporation or ultrasonic nebulizer-free options. The concentrations of several proteins including horse heart cytochrome c, were determined by using the ICP-AES method and found to compare favorably with concentrations obtained by using UV-visible molar absorptivities based on traditional methods of protein determination [18 and references therein]. In addition to sulfur analyses, iron analyses can be run on the same protein samples using the Fe(II) emission line at 248.30 nm

[49]. Analysis of native horse heart cytochrome c, after initial desalting on the ion-exchange column, gave a ratio of Fe/protein of 0.94 ± 0.03 . ICP-AES analyses gave a protein concentration of $47.3 \pm 1.2 \mu\text{M}$ as compared to the UV-visible determined protein concentration of $48.8 \mu\text{M}$ by using the absorption band at 410 nm ($\epsilon = 106,100 \text{ M}^{-1} \text{ cm}^{-1}$) [50].

While ICP-AES analyses of the cytochrome c reaction products will determine the extent of protein modification, the analyses will not show the site of modification. The site of cytochrome c modification can be determined by two methods. In the first method, tryptic digestion of the protein and comparison of chromatographic mobility of the fragments with those of the native protein is used to determine the modified fragment. Once the modified fragment has been identified, MSN analysis of the fragment will identify the site of modification. In the second method, ^1H MSN spectrum of the modified protein will reveal if the Met 48 or Met 51 side chains have been modified. As mentioned in Milliken paper, alkylation of Met 48 causes the sharp methyl resonance at δ 1.1 to shift downfield by 0.75 ppm forming a distinct shoulder in the cytochrome ^1H MSN spectrum [45]. The integrity of the heme environment can also be assessed by ^1H MSN experiments by monitoring the region in the MSN spectrum of the modified protein between δ

8 and 8 +4 where the methylene and methyl resonances of Ref 8b are located [16].

Results and Discussion

Model Studies

In order to determine the optimum reaction conditions for the new ferrocenyl-alkylating reagents we undertook ¹H NMR model studies using ethylmethylcarbazide (EMMC) as a model for the thioether of methionine. Methionine could not be used as it contains an amine group that is also available for alkylation. In addition, the ferrocene derivatives are not very water-soluble and methionine is not very soluble in solvents that dissolve the ferrocene derivatives.

Upon alkylation of the model compound, a positive chemical shift difference of 1.1 ppm was noticed for both the thioether methyl singlet (a-a') and methylene quartet (b-b') of EMMC, while the EMMC beta methyl triplet (c-c') had a positive chemical shift difference of 0.1 ppm (Figure 1-6). The haloalketyl methylene undergoes an exchange reaction with the deuterated methanol solvent, and thus a peak is not observed. Upon creation of the sulfonium ion, the methylene protons become magnetically inequivalent (see Figure 1-7), thus a overlapping doublet of quartets is observed rather than a simple shifting of the methylene

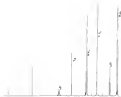


Figure 2.4. A typical ^{59}W NMR spectrum of an oligoalkyl tungsten $[\text{C}_6\text{H}_5\text{O}(\text{C}_4\text{H}_9)_2\text{W}]_n$ that has reacted with H_2SO_4 to produce the corresponding sulfonate ion.



Figure 3-7. Newman projection of the sulfonium salt methylsulfonium hexafluorophosphate showing the inequivalence of the methylene protons.

quartet. The results of model studies with the known organo-, amino isocyanide, and ferrocenyl alkylating reagents are presented in Table 2.1.

Halocetylferrocene (FcHCl) and halocarbonyl-ethylferrocene (FcHClCEt) did not react with RNA in 50% at 40°C, even after three days. Temperatures higher than 40°C would denature cytochrome c, so further studies were not undertaken with these reagents. FcHCl may not react with the model compound because the electron donating ability of the ferrocene moiety may decrease the electrophilicity of the halocetyl functional group. Direct electronic effects are not likely for the FcHClCEt, which has two saturated carbon atoms between the ferrocene moiety and the functional group. FcHClCEt may be less electrophilic than its organic analog due to other possible interactions between the ferrocene moiety and the functional group, since the functional group is not held rigidly away from the iron center; however, the carbonyl stretching frequency and ^{13}C NMR shifts of the carbonyl do not indicate such an interaction. p-bromobenzenesulfonylferrocene (FcHBrSO₂Ph) was prepared to investigate the above mentioned interactions. The phenyl ring holds the halocarbonyl functional group rigidly away from the ferrocene moiety and allows us to probe the effect of the electron donating ability of the ferrocene moiety. Ferrocene has a para Hammett sigma constant of -0.16 [73], which indicates that

Table 3.3.
Chemical Shift Differences Between DMSO and Its
Corresponding Sulfoxide Derivatives.

SOURCE	SULFOXIDE	NMR Shifts of DMSO upon Alkylation		
		sec ¹	tert ²	quat ³
Signs	SO ₂ COOH	1.1	1.3	2.3
Aldrich	C ₆ H ₅ C(=O)CH ₃ CH ₃	1.1	1.3	2.3
synth.	SO ₂ CH ₂ CH ₂ CH ₃	1.0	1.0	2.3
synth.	SO ₂ CH ₃	no reaction observed		
synth.	SO ₂ CH ₂ CH ₃	no reaction observed		
synth.	SO ₂ CH ₂ CH ₂ CH ₃	1.3	1.3	2.3

All reactions were run on a Varian V60-60 instrument operating at 100.620 MHz for protons. The solvent, CD₃OD was used for all reactions. Shifts were referenced to CD₃OD at δ 3.30. Chemical shift differences are reported in ppm. All reactions were at room temperature (ca. 25°C) except for SO₂COOH and C₆H₅COOCH₃CH₃ (55°C).

the ferrocene moiety is approximately as electron donating versus a phenyl ring as a methyl group (-0.17) [73]. A second reagent was, therefore, prepared combining the phenylferrocene derivative, but having a methyl group para to the sulfonamide functional group forming p-toluenesulfonamide (Ptothensulfide). Ptothensulfide has been used by Strauss and Linsen [66] to modify the methionine residue of Chymotrypsin and this should be reactive with EDC and the Met 83 of HbC α . Indeed both Ptothensulfide and Ptothensulfide react with EDC.

By using at least a ten-fold excess of EDC, two experiments were run to determine the alkylation rate constants for the reactions of EDC with Ptothensulfide and Ptothensulfide. Integrals of the sulfonamide methyl triplet and singlet were plotted against time to yield a curve. $\ln(A - A_0)$ vs time (where A represents the integral of the sulfonamide methyl singlet or triplet) was plotted to obtain a straight line giving an average pseudo first order rate constant of $3.1 \pm 10^{-4} \text{ s}^{-1}$ and second order rate constant of $k = 2.07 \pm 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ for Ptothensulfide and average values of $k_{\text{obs}} = 5.5 \pm 10^{-4} \text{ s}^{-1}$ and $k = 4.4 \pm 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$ for Ptothensulfide (see Tables 2.1 and 2.2 and Figures 2.4 - 2.12).

Table 3.3.

Kinetic data for the reaction of EtOH with
 H₂PO₃ANACHE. Reaction was run in CD₃CO at 50°C.

$$[\text{H}_2\text{PO}_3\text{ANACHE}] = 1.75 \times 10^{-3} \quad [\text{EtOH}] = 1.134 \times 10^{-2}$$

time/min	Sulfonium Ion Methyl Triplet		Sulfonium Ion Methyl Singlet	
	$\Delta\text{intensity}$	intensity	$\Delta\text{intensity}$	intensity
3	0	0.00	0	0.00
5	4.0	0.00	4.7	0.00
10	8.0	0.00	8.4	0.00
20	9.0	0.00	11.0	0.00
30	13.0	0.00	15.9	0.00
40	15.0	0.07	18.0	0.00
45	17.0	0.08	19.0	0.07
50	18.0	0.08	20.1	0.08
100	19.0	0.70	20.0	0.00
110	19.7	0.51	20.4	0.07
120	20.0	0.03	24.3	0.04
140	20.4	-0.00	24.0	-0.00
160	20.8	-0.00	25.0	-1.00
180	21.1	0.00	25.3	0.00

Table 2-4.

Kinetic data for the reactions of DMSO with
 Tetramethylethylene in DMF at 50°C. Data was obtained by
 measuring the height of the solvent singlet of the calibration
 ion at 18 gauss/cm.

1. $[\text{Tetramethylethylene}]_0 = 710 \text{ mM}$; $[\text{DMSO}] = 4.2 \text{ mM}$

<u>Time(min.)</u>	<u>Peak.Ht.</u>	<u>time</u>	<u>conc.(mM)</u>
0	0	—	—
24	7	31	3.8
48	18	31	3.4
64	31	31	3.4
88	44	36	3.8
148	68	67	3.2
180	88	31	3.7
188	94	31	3.9
220	108	31	3.2
251	129	31	4.2

2. $[\text{Tetramethylethylene}]_0 = 670 \text{ mM}$; $[\text{DMSO}] = 7.4 \text{ M}$

<u>Time(min.)</u>	<u>conc.(mM)</u>	<u>time</u>	<u>conc.(mM)</u>
0	0	—	—
27	4.5	34	6.2
71	23.2	34	18.9
100	34.9	34	21.7
133	74.7	66	19.8
191	111.1	177	18.8
268	137.4	64	14.5
450	159.4	64	11
718	213.8	294	14

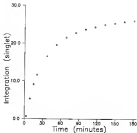


Figure 3.8: Kinetic plot for the reaction of 20% with 2,4-dinitrochlorobenzene at 50°C using the integration of the sulfonium ion singlet (J 3.60) vs time.

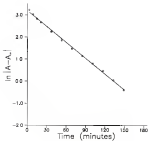


Figure 2.9. Plot of $\ln|A - A_{\infty}|$ versus time for the reaction of Methoxybenzoyl with EtOH at 25°C . A = integral of the sulfonate methyl resonance at δ 3.88, A_{∞} = integral of the methyl peak at infinite time).

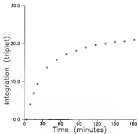


Figure 2.18. Plot of Integral versus time for the methyl triplet (J 1.8s) of the sulfonium ion prepared from the alkylation of DMSO by methanesulfonyl at 30°C

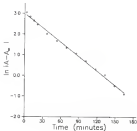


Figure 2-11. The plot of $\ln |A - A_{\infty}|$ vs t for the reaction of perfluorooctane with styrene using the ethoxycarbonyl methyl triplet... A is the integral of the methyl resonance, A_{∞} the integral of the methyl resonance at time infinity.

Modification of Borna Bort Cytochrome c

Williams' results have been reproduced with both iodoacetic acid and iodoacetamide. In both ^1H NMR experiments, the sharp resonance of the set of 45 thioethers were no longer visible in the NMR spectrum of the modified cytochrome c. The iodoacetamide modified NMRc elutes from a cation exchange column at a higher ionic strength than native cytochrome c indicating that the modified cytochrome c has a higher positive charge than the native protein. The modified protein is expected to have an additional positive charge from the sulfonium ion created upon alkylation of methionine 45. Thus far, none of the reactions of the ferrocenyl alkylating reagents with cytochrome c have yielded a modified cytochrome. The organic solvent used to solubilize the ferrocene derivative has been changed from ethanol to acetonitrile, and the pH has been varied from 2.5 to 7. Reaction times from 1 hour to 28 days have been employed. The most promising ferrocenyl derivative [FcPhosMAAde], based on the results of model studies, is very water-insoluble making it difficult to modify the protein considering that the reactants are in two different physical states.

An attempt to solubilize the FcPhosMAAde was made following the procedures used by Friedlander (74) and yielded a yellow water-soluble powder when precipitated with

Fe_4^{2+} . The yellow water-soluble powder had an electronic absorption band at 388 nm and absorption cut to 444 nm. The compound was not further characterized. Since an electron transfer reaction with the oxidized ferrocene derivative and ferrocyanide could result in the reduced ferrocene derivative precipitating out of solution, cytochrome c was used in its oxidized form for the alkylation reaction. No modification was observed.

Cytochrome c has been modified with an average of one mole of ferrocenecarboxymethyl acid (FeCOOH) bound per mole of cytochrome c by using the carbodiimide promoted coupling reaction described in chapter 3. In a second reaction, urea was used to slightly open the cytochrome c structure by disrupting the hydrogen bonding that helps to hold the quaternary structure in place. Again, the cytochrome c was modified with an average of one ferrocene bound per mole of cytochrome c. Cation-exchange chromatography revealed that the cytochrome c was heterogeneous with respect to the site of modification. Ion-exchange fractions of the cytochrome were too small for further analysis to determine the various sites of modification. HEC contains 11 lysine residues, which are the primary targets for the carbodiimide promoted modification.

Experimental section

Model studies with STMs

In general, a 5 mlol excess of ethynethynolide was transferred with a calibrated pipet into a vial containing the alkylating reagent. 400 μ l of CH_2Cl_2 was added and the solution was transferred into an NMR tube. The alkylation was quick enough that the spectrum could be taken within 10-20 minutes. However, for the alkylation reactions of Folsol and Folsolene with STMs, the NMR tube was sealed with parafilm and the reaction solution was kept in a 30°C water bath for 12 hours yielding no alkylated product. The reactions were repeated in deuterated benzene to insure the precipitation of cationic products. The precipitation gives the reaction an additional driving force. No alkylated product was produced.

Reaction of Methacrylate with STMs. Methacrylate (20.1 mg) was dissolved in 100 μ l of CH_2Cl_2 and placed into an NMR tube. STM (79.8 μ l) was added to 100 μ l of CH_2Cl_2 and placed into a vial. An initial spectrum was run in order to obtain the proper receiver gain, lock, and spin setting. After the instrument was set up, the STM solution was transferred into the NMR tube and mixed with the reactant solution. The deuterium lock was re-

established and the field homogeneity was fine-tuned. Three minutes passed between the initial introduction of the EPRB solution and the initial scan. Each spectrum was acquired with 10 transients at 50°C. The three sulfonate peaks and the methyl peak of bromocycanilide were integrated.

Integrals of the sulfonate methyl triplet and singlet were plotted against time to yield a curve. $\ln(A/A_0)$ vs time was plotted to obtain a straight line giving an average pseudo first order rate constant of $2.3 \times 10^{-2} \text{ min}^{-1}$ and second order rate constant of $k = 2.87 \times 10^{-5} \text{ M}^{-1} \text{ s}^{-1}$.

Reaction of PufPhenylsulfate with EPRB. PufPhenylsulfate (3.0 mg) was dissolved in 500 μl of CH_2CO and placed into an EPR tube. After the instrument was set up, as described for the Methoxyaniline reaction, 3.3 μl of EPRB in 500 μl of CH_2CO was quickly added to the bromocycanilide solution, mixed, and replaced into the EPR. The experiment was run, as described for the Methoxyaniline reaction at 50°C. The initial spectrum was started 3 minutes after the introduction of the EPRB reaction. By using the peak heights of the sulfonate methyl singlets and the integral of the phenyl protons as a measure of the initial concentration of PufPhenylsulfate, we calculated the pseudo first order rate constant to be $2.3 \times 10^{-2} \text{ min}^{-1}$ and the second order rate constant to be $3.8 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$. The experiment was repeated using 1.8 mg of PufPhenylsulfate and 4.18 μl of EPRB in 500 μl of CH_2CO , but over a longer period

of time (13.8 hours) and with 84 scans/spectrum. The pseudo-first order rate constant obtained from the integration of the valideron methyl doublet peak was $1.1 \times 10^{-4} \text{ min}^{-1}$ and the second order rate constant was $1.2 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$ giving $4.8 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$, as an average second order rate constant for the two runs.

General Procedure for Protein Immobilization

Many attempts have been made to alkylate the Met 43 residue of MBP with the Marrowgl alkylating reagents. This section outlines the general procedures employed for protein modification. Normal heath cytochrome c was obtained from Sigma (type VI) as a lyophilized powder, oxidized with a 4-fold molar excess of $\text{Fe}(\text{OH})_3^{+}$, and then desalted [78] on a Sephadex CM 2B-120 cation exchange column. The 10 column was equipped with 1000 fraction collector, absorbance monitor, and peristaltic pump. The pump rate was 10 ml/hr, and 1 ml fractions were collected. UV absorbance was monitored at 210 nm. Fractions having absorbance at the proper elution volume were pooled and concentrated to 1 ml by using an Amicon ultrafiltration apparatus (UM 5). The concentrated protein solution was placed into a dialysis bag (pore cutoff 8-9000) and the dialysis bag placed into a dialysis flask equipped with an argon bubbler. The dialysis flask was filled with argon-saturated phosphate buffer and

excess dithionite reagent to reduce the heme iron center to the ferrous state in order to strengthen the Fe-Hist 88 bond and decrease the amphiphilicity of Hist 88 (40). Once the protein was fully reduced, the dithionite solution was concentrated out of the dialysis flask leaving the protein dialysate bag intact under a slight pressure of argon. The cherry-orange protein solution was concentrated into a solvent tube previously flushed with argon for 20 minutes. A slight vacuum was pulled on the protein solution so that the protein could be safely taken into the K_1 box through the antechamber. In addition to the protein solution, buffers, acetonitrile, ethanol, and ferrocene reagent were taken into the K_1 box after previously saturating the buffers with argon and taking the organic solvents through three freeze-pump-thaw cycles to remove dissolved oxygen. Once inside the box, the protein was washed in the ultrafiltration apparatus with two 50 mL portions of phosphate buffer to remove the remaining dithionite reagent, followed by two portions of the appropriate reaction buffer (typically, 1 M acetate buffer, pH 5.4) to protonate the side chains of the unreacted reaction sites. The ferrocene derivatives (100-fold excess) were dissolved (ca. 2 mL) of acetonitrile (or ethanol) and then were added to the protein solution, which had been concentrated to 2.5 mL. Some reactions were allowed to proceed in the box at approximately 25°C, while others were run in subbank tubes in a water bath at 25°C and

returned to the box for wash up procedure. When the reaction was finished, the protein was washed with two 50 ml portions of the reaction buffer followed by two 50 ml portions of phosphate buffer, concentrated to about 1 ml and applied to a Sephadex G-65 size-exclusion column. Fractions containing cytochromes were pooled and concentrated to 1 ml then oxidized with a four molar excess $\text{Fe}(\text{Phen})_3^{2+}$. Tris-phenanthrolinecobalt(III) perchlorate was used as an oxidant for the potentially modified proteins rather than ferricpyridide, which was found to form charge-transfer complexes with ferrocene derivatives [74]. Oxidized fractions were applied to the CM 10-120 ion-exchange column and chromatographed by using a gradient of 50mM phosphate to 0.5 M HCl-phosphate. The elution of the Met 45 residue produces a sulfonium ion, which adds a positive charge to the total charge of the protein, thereby giving the modified proteins a different chromatographic mobility on a cation-exchange column than the untreated proteins.

Reaction of $\text{HNO}_2(\text{Cl})$ and $\text{FeSO}_4(\text{Cl})$ using dialysis. $\text{HNO}_2(\text{Cl})$ (12 mg, Sigma, type VI) was dissolved in a Sephadex CM 10-120 cation exchange column (1.4×25 cm) by using a gradient of 50 mM phosphate buffered at pH 7.1 to 0.5 M HCl in phosphate and a pump rate of 10 ml/hr. The absorbance was monitored at 335 nm. Fractions 14-18 containing 2 ml each were collected and concentrated to 1 ml by using an

ultrafiltration apparatus (Salcon 20 S). The protein was placed into a dialysis bag (BB cutoff 6000-8000) and then placed into the dialysis flask equipped with an argon inlet. Under a slight pressure of argon, the protein was dialyzed overnight against 100 ml of 10 mM acetate solution buffered at pH 5.0. The acetate buffer was replaced, via cannula, by an argon-saturated solution containing excess dithionite, which reduces the heme iron. After eight hours, the dithionite solution was replaced twice by argon-saturated acetate buffer removing residual dithionite, which would reduce any sulfonium ion formed during our alkylation experiment and equilibrated for 14 hours per replacement solution. The acetate solution was replaced, via cannula, by argon-saturated reaction solution, which contained 10 mg of Potassium dissolved in 100 ml of 10% ethanol-acetate buffer and allowed to react for 48 hours. The dialysis solution was replaced, via cannula, twice with 50 ml portions of argon-saturated acetate buffer (6 hours equilibration time each) to remove any remaining Potassium and then with a 50 ml portion of argon-saturated phosphate buffer (14 hours equilibration time). The reaction was terminated by applying the protein solution to a Sephadex 6-75 size-exclusion column. Fractions having absorbance at the proper elution volume were concentrated to 2 ml. The protein was oxidized with a four molar excess of ferricyanide and was applied to the cation exchange column.

ICP-MS analyses of the single cation exchange peak indicated that the protein was unmodified.

Reaction of BSA with PMSF. BSA (25 mg) was dissolved. The protein was reduced via dialysis against dithionite in argon-saturated 50 mM phosphate buffered at pH 7.4. The dialysis solution was changed twice with 100 ml portions of the argon-saturated phosphate buffer and allowed to equilibrate. The protein solution was transferred, via cannula, into a Schlenk tube previously flushed with argon. A slight vacuum was pulled on the protein solution so that the Schlenk tube could be safely taken into the N_2 box. Inside the oxygen-free box, the protein was washed in the ultrafiltration apparatus (Amicon, YMN) with two 50 mL portions of 1 mM acetate buffer (pH 5.0) and concentrated to 1 mL. The protein solution (1.5 mL) was placed into a Schlenk tube containing 10 mL of argon-saturated 10% ethanol-acetate solution, as a blank. The second 1.5 mL protein solution was placed into 11 mL of argon-saturated 10% PMSF acetate solution containing 1 mL of ethanol and 1 mL of 1 mM acetate buffer (pH 5.0). The reactions were allowed to proceed for four hours. Both protein solutions were washed in an ultrafiltration apparatus with two 50 mL portions of 1 mM acetate buffer and two 50 mL portions of 50 mM phosphate buffer (pH 7.4), respectively. The protein solutions were concentrated to about 1 mL and applied to a

Sephadex G-50 size-exclusion column. A single cytochrome band was collected for each reaction. The G-50 fractions were pooled and concentrated to about 1 ml. The proteins were oxidized by ten equivalents of ferricyanide and then applied to the cation exchange column. A single peak was observed for each reaction. ICP-MS analysis indicated that the protein had not been modified by Potassium and that the protein did not lose iron under the experimental conditions. The reaction was repeated twice allowing the reaction to proceed for 72 hours and for 20 days; however, the protein was not modified in either case.

Reaction of Cytochrome c with Potassium. Following the procedure used for the latter set of reactions of Potassium with Hbcs, 15 mg of Hbcs was prepared for the reaction omitting the desalting step. 48.6 mg of Potassium (48.6 mg) was dissolved in 1 ml ethanol then added to 8 ml of 1 mM acetate buffered at 5.5 both solutions were previously saturated with argon. The protein was added to the ferrous solution, allowed to react for four days, and then worked up according to the procedure outlined for the later reactions of cytochrome c with Potassium. ICP-MS revealed that the protein was unmodified. The reaction was repeated with 35 mg of Hbcs with the same results.

After finding that ferricyanide can form a charge-transfer complex with ferrous derivatives, the cytochrome

α -Fetakeal reaction was repeated using $\text{Co}(\text{Phen})_3^{2+}$ as the final protein oxidant. $\text{Co}(\text{Phen})_3^{2+}$ was prepared according to the procedure of Taylor (11). HbCo(II) (47 μg) was prepared as previously described. One-quarter of the protein was used in a control experiment, the rest was added to a solution containing 7.4 μg of Fetakeal in 1 ml. acetic acid and 1.5 ml of acetate buffer giving a total reaction volume of 10 ml. The reaction was allowed to proceed for one week then working-up as mentioned before, but substituting $\text{Co}(\text{Phen})_3^{2+}$ for the ferricyanide. The ion-exchange chromatogram contained four peaks. ICP-AES analysis indicated no protein modification. The reaction was repeated five times using 4 μg of protein for each reaction and varying the pH of the reactions to pH 7.0 (50 mM phosphate), pH 5.0 (acetate 1M), pH 4.5 (1 mM acetate), pH 4.0 (1M acetate), pH 3.5 (1 mM acetate) and using a total reaction volume of 3.4 ml. The reactions were worked up, as mentioned before, with no protein modification indicated by reliable ICP-AES analyses.

Experiments of HbCo with Ferricyanide. HbCo (31 μg) was prepared as described for the earlier reactions of HbCo. After washing the protein with acetate buffer (in the H_2 box), the protein was washed with a 10 ml portion of deionized water to reduce the concentration of acetate to 0.1 mM. The protein was placed into a schlenk tube, removed

from the N_2 box, and freeze-dried on a schlenk line for 24 hours. The freeze-dried powder was taken into the N_2 box. One-half of the powder was dissolved in 500 μ l of H_2O , which had been previously degassed via three freeze-pump-thaw cycles. Porphobilinogen (3.4 mg) was dissolved in 100 μ l of CH_3OH (previously saturated with argon) and placed into a vial. The vial was sealed with a rubber septa that was held in place by copper wire. An initial spectrum of the reduced cytochrome c was taken and the instrument was set up for an arrayed experiment and for a temperature of $10^\circ C$. The Porphobilinogen solution was cannulated under N_2 into the KNO_3 solution. A precipitate was observed. No modification was observed. IEF-AGE analysis of the protein (after the work up described above for the reaction of KNO_3 with PMSA) (Na_2Phos_3 as the oxidant) confirmed that no modification of the protein had taken place.

In a second attempt at modifying the protein with Porphobilinogen, Porphobilinogen (3.1 mg) was placed into a schlenk tube, taken into the N_2 box, and dissolved in 400 μ l of CH_3OH . The KNO_3 (22 mg) prepared for the earlier reaction of KNO_3 with Porphobilinogen was dissolved in 400 μ l of the acetate buffer and added to the schlenk tube containing the ferrocene solution. The schlenk tube was sealed, taken out of the box, and placed in a water bath ($40^\circ C$) for 24 hours. The schlenk tube was returned to the N_2 box and the protein solution was worked up as described

previously. HRP-AGE analyses indicated that the protein was not modified. The reaction was repeated using BSA (1000 mg) and P-Phenanthroline (9.7 mg, some of the derivative did not dissolve and was filtered out before the protein solution was added). Phosphate buffer (pH 7.2, 45 mL) was used instead of the acetate buffer. The Schlenk tube containing the reactants was placed into a water bath (40°C) for 16 hours, and then worked up as previously described. HRP-AGE analyses indicated that the protein was not modified.

Reaction of BSO₂ with Iodoacetamide. BSO₂ (80 mg) was prepared as described for the earlier reactions of BSO₂. After washing the protein with acetate buffer (in N_2 box), the protein was washed with a 20 mL portion of deionized water to reduce the concentration of acetate to 0.25 M. The BSO₂ solution was concentrated to 1 mL. Iodoacetamide (97.5 mg) was dissolved in 2 mL of water and placed into a Schlenk tube. The pH was adjusted to 8.8. The Schlenk tube was sealed. The solution was degassed by using three freeze-pump-thaw cycles before bringing it into the N_2 box. The BSO₂ and iodoacetamide solutions were combined and allowed to react at room temperature for 4 hours. The protein solution was worked up as described for earlier reactions of BSO₂. A single peak eluted from the Sephadex G-50 size-exclusion column at the elution volume for the native protein. Two peaks eluted from the CM10-120 cationic-

exchange column using a linear gradient of 0.0-0.5 M KCl (500 ml of solvent in each gradient flask). The first peak eluted where the native protein typically elutes. The second peak eluted after the native peak at 180 ml indicating a higher positive charge on the second protein fraction. The fractions corresponding to the second peak were pooled and concentrated to 1 ml and freeze-dried. The protein powder was dissolved in 200 μ l of D₂O for NMR analysis. After the initial spectrum was obtained, a few small crystals of one reference compound were added to the sample so that the spectrum could be properly referenced.

Reaction of HSCA with FeCl₃. HSCA (18 mg) recovered from earlier reactions were combined and concentrated to about 1 ml and dialyzed. The low-exchange fractions were pooled and washed with three 50 ml portions of 0.1 M Tris buffer (pH 7.3) and concentrated to about 1 ml. The protein solution was added to a solution containing DCC (25 mg) and FeCl₃ (50 mg), previously dissolved in 1 ml of Tris buffer for 15 minutes, and was allowed to react for 24 hours at 4°C and then at room temperature for 24 hours. A colored pseudophen was noticed and was filtered out (Baker, 0.45 μ m) before the protein was worked-up. After the reaction, the protein was washed with 50 ml of deionized water via ultrafiltration (Baker, 10K) to remove any unreacted coupling reagent, which might bind to the amino

oxidation column, concentrated to about 1 ml, and applied to the Sephadex G-50 size-exclusion column. A single peak was collected. ICP-AES analysis of a portion of the G-50 fraction indicated an average of 2.83 ± 0.58 moles of iron per mole of HbCo indicating a single mole of ferrocene per mole HbCo. The remaining portion of the G-50 fraction was concentrated to about 1 ml, oxidized with a 4 molar excess of $\text{Co}(\text{Phen})_3^{2+}$, and applied to the Sephadex CMB-120 cation-exchange column. By using a gradient of 0 M KCl in phosphate buffer to 0.18 M in phosphate buffer, 5 fractions of cytochromes were eluted. The cytochromes c samples were too small for ICP-AES and tryptic digestion analyses.

Reaction of HbCo with Ferrocene and Urea. The procedure for the reaction of HbCo with Ferrocene was followed with the exception that the reaction mixture also contained 250 mg of urea. ICP-AES analysis of the single G-50 fraction revealed that an average of 2.83 ± 0.58 moles of iron per mole of cytochromes indicating an average of 1-2 moles of ferrocene per mole HbCo. Cation-exchange of the remaining portion of the G-50 fraction contained four cytochromes peaks. The cytochromes c samples were too small for ICP-AES and tryptic digestion analyses.

CHAPTER 3
MULTI-SITE MODIFICATION OF SWINE SIVIN ALBUMIN AND HUMAN
LIPET BISOCTATE

Introduction

The long-term goal of the multi-site modification project is to prepare and characterize monoclonal antibodies against metal complexes. Antibodies specific to transition metals chelated by ethylenediaminetetraacetic acid (EDTA) have been developed as radiotracers (74-80). Since ferric ions elicit a strong immune response (81), a series of ferric complexes have been prepared for the development of metalloprotein specific antibodies. Such metalloprotein specific antibodies can be used for a number of projects including the development of radiotracers and synthetic enzymes. This chapter outlines potential applications of the metalloprotein specific antibodies, the method developed for multi-site conjugation of ferric complexes to carrier proteins, the method developed for determining the extent of protein modification, and the results of hybridoma studies. The preparation and characterization of the ferric and ruthenium containing derivatives are presented in chapter 4.

Potential Applications of Metalloporphyrin Specific Antibodies

Kosman and co-workers have produced antibodies against a transition metal complex (TMC) [74, 75]. The antibody against the transition metal complex is conjugated with a cancer specific antibody to form a hybrid antibody that is specific for both the metal complex and the cancer tumor. The hybrid antibody is reacted with the TMC containing an appropriate radioactive isotope of the metal, and then introduced into the patient. The antibody portion of the conjugate directs the conjugate to the cancerous site, which can then be more clearly detected. While EOTX and EPX conjugates are inexpensive and easy to prepare, unfortunately, they are generally toxic. Metalloporphyrins offer the advantages of the chlorin a system and may be less toxic.

As described in more detail in chapter 1, several groups have been investigating the potential use of ruthenocene and ferrocene derivatives as radiotracers [14-18]. In general, these metalloporphyrins have proven to be non-toxic, stable in vivo, and excreted within 24 hours; however, metalloporphyrins are not very water soluble. By conjugating a mildly radioactive form of the metalloporphyrin with specific antibodies like those described for the TMC, a more specific and water soluble metalloporphyrin radiotracer can be produced. In addition to being non-toxic, the metalloporphyrins have a neutral charge that may allow the

conjugate to penetrate hydrophobic areas and to concentrate in the cancerous area without building up charge that would inhibit further conjugation) [10].

As described in detail in chapter 3, another potential use of metallocones is in the development of synthetic enzymes. Benson and co-workers have modified cytochrome with furanose derivatives to produce a synthetic enzyme capable of hydrolyzing *p*-tert-butylphenylacetate at a rate 1.5×10^3 times greater than without the synthetic enzyme and represents the largest acceleration of a reaction by a synthetic enzyme thus far [11].

Field of Metallocone Modification of Carrier Proteins

The development of antibodies to small haptens, such as furanose, requires the conjugation of the small hapten to a large carrier protein of no less than 10 kDa. Typically bovine serum albumin (BSA, MW 47.5 kDa) and keyhole limpet haemocyanin (KLH, MW 800 kDa) are used as carrier proteins, which are necessary to stimulate the antibody producing B and T cells of the immune system. Many types of antibodies will be produced against the carrier protein conjugate including those which are specifically against the metallocone moiety. At least twelve antigens are typically bound to the carrier proteins to produce a sufficient supply of the metallocone specific antibodies for screening and

collection. In addition, to ensure production of antibodies that are specific for the metalloenzyme in its unconjugated form, a spacer group of 4-8 atoms between the carrier protein and the metalloenzyme moiety may be helpful. In order to further aid in the screening process, the antigen is generally conjugated to more than one carrier protein to screen out those antibodies that are against the carrier proteins rather than hapten.

The carrier proteins most commonly used for this type of immunological work are BSA and KLH. BSA is a plasma protein composed of a single polypeptide chain containing 580 amino acid residues and has a molecular weight of 66.2 kDa as determined by size-exclusion chromatography [88]. The protein is compact and nearly spherical. It contains 17 disulfide bridges and one sulfhydryl group in addition to 4 methionine residues giving a total sulfur content of 19 moles of sulfur per mole of BSA [88]. Of the 580 amino acid residues in BSA, three have still not been unequivocally determined, a proposed glycine residue has been suggested to be possibly a cysteine residue [89], which will become important later in this chapter for the quantification of the extent of modification of BSA.

The second carrier protein, KLH, is a limulus copper oxygen-binding glycoprotein found in the hemolymph of several marine and terrestrial invertebrates belonging to Mollusca and Arthropoda [90]. When deconjugated,

hemocyanins are colorless upon binding of one mole of dioxygen with two moles of copper. The proteins become blue. Hemocyanins are high molecular weight proteins (up to 10^7 Da), which can easily dissociate into subunits. The dissociation-association reaction depends on the protein concentration, ionic environment, pH, and other factors. Amino acid compositions are known for several molluscan and arthropod hemocyanins; however, no complete amino acid sequences are known for any of the hemocyanins. Hemocyanins are classified into two groups: molluscan hemocyanins and arthropod hemocyanins due to their similarities in number and weight of subunits and by their similarity in copper content. Of particular interest to this study is the molluscan hemocyanin, obtained from *Mytilus edulis*, *Mytilus* *galeatus*, which has 10 subunits per mole of subunit at neutral pH (8-9). This is used as a carrier protein possibly due to the stability of the associated protein relative to its subunits at neutral and alkaline pH.

A series of ferrocenyl ketosides of the type $\text{Fc}(\text{CH}_2)_n\text{OR}$ (where $n=0,1$) were prepared according to the procedure of Hunsburt [85]. Specific modifications to his procedure are presented in chapter 4. The various suitable ferrocene derivatives may be conjugated to the carrier protein via the carbodiimide coupling method described in chapter 1 for the ferrocene modified glucose oxidase electrode system (see Figure 3.1). In step 1 of the

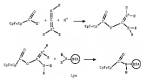


Figure 1.1. General Mechanism of Carbodiimide Coupling.

reaction scheme in Figure 3.1, the ferrocene carboxylic acid reacts with the carbodiimide coupling reagent producing the *o*-acylisourea intermediate. In step 2, the intermediate undergoes a condensation reaction with the epsilon side chain of lysine to produce the corresponding amide bond thus covalently attaching the ferrocenyl carboxyl group to the protein.

Results and Discussion

The carrier proteins BSA and HSA have been modified with FCBUTs by using the water-soluble carbodiimide coupling reagent, NDC. BSA was modified with 10 moles FCBUTs/moles BSA and HSA was modified with 1.5 moles FCBUTs/moles HSA. Both modified proteins show an increase in their absorbance to 560 nm, with the sharp peaks of the native protein being nearly obscured by the increase in absorbance in this region of the UV-visible spectrum due to absorbance from the ferrocene derivative. ICP-MS analyses of the modified BSA to determine the extent of modification gave very close results to those obtained by spectrophotometry after correcting the 194 nm absorbance for tyrosine modification. FCBUTs was the first ferrocene derivative to be conjugated to the carrier proteins and undergo immunological studies because it has the largest number of spacing units between the protein and the ferrocene moiety. Initial immunological

studies indicate that enzyme-specific antibodies were not produced.

Experimental Section

General Procedure for Notifying EED and EED

The proteins were obtained from Sigma Chemical Co. as lyophilized powders and further purified on a Sephadex size-exclusion column (G-200 for EED and G-200 for EED). 0.5 M KCl in phosphate buffer (50 mM, pH = 7.5) was the eluent at a pump rate of 30 and 7 mL/hr, respectively. 5 and 5.5 mL fractions were collected. The fractions having UV absorbance at the appropriate elution values, as detected by an Inco UV detector set at 225 nm, were pooled, washed twice with 50 mL portions of the 0.5 M $\text{NaHCO}_3/\text{KCl}$ buffer (pH = 10.5), and then concentrated to 1 mL. The protein solution was then pipetted into a solution containing the ferrocene derivative and EED dissolved in 1 mL of the $\text{NaHCO}_3/\text{KCl}$ buffer.

The ferrocene derivative is used in 100% excess and the carbodiimide is used in 100% excess relative to the protein concentration following Bogard and Miller's preparation. The excess of ferrocene over the coupling reagent ensures complete formation of the ferrocenyl-acyliminium intermediate so that no coupling reagent is unused before addition of the protein, where the reagent would polymerize

the protein. The reaction was allowed to proceed for 48 hours at room temperature. Next, the reaction mixture was filtered (Nalson, 4-10 μ) to remove any precipitate formed during the reaction, washed twice with 50 ml of the phosphate eluent buffer to remove any unreacted ferrocene and DCC, and then concentrated to 1 ml and applied to a Sephadex G-200 or G-200 size-exclusion column. The higher molecular weight fractions collected were groups and corresponded to the modified protein. These fractions were collected and concentrated (leaving the ultrafiltrate as a blank) for analysis by UV-visible and NCP-MS. The remaining modified protein was washed with four 50 ml portions of deionized water to remove HCl, which might have the side used for the immunological studies on the protein. The modified protein was concentrated to 1 mg protein/ml and submitted for immunological studies. Initial immunological results indicate that no production of antibodies specifically against the metalloenzyme-protein conjugate has taken place.

Modification of BSA with 5,5'-DITHIOBIS(2-NITROBENZYL CARBOXYLATE). BSA (21 mg, Sigma, 4-7819) freeze-dried protein was dissolved in 2 ml of the eluent phosphate buffer and then desalted on a pre-equilibrated Sephadex G-100 column. The UV chromatogram showed only one peak corresponding to the appropriate elution volume for BSA indicating little or no impurity.

Fractions corresponding to the elution volume of the protein were collected and washed twice with 50 mL of the $\text{NaHCO}_3/\text{OH}^-$ buffer and then concentrated by ultrafiltration (Amicon, 100) to approximately 1 mL. $\text{CpHcyCpO}(\text{O})(\text{CH}_2)_2\text{COOH}$ (31 mg, 0.048 mmol) and BSA (43 mg) were dissolved in 1 mL of the $\text{NaHCO}_3/\text{OH}^-$ buffer. The protein solution was added to the ferrocene solution and then allowed to react for 48 hours at room temperature. The reaction mixture was then filtered (Whatman, 0.45 μm) to remove the precipitate formed during the reaction, washed twice with 50 mL portions of the phosphate buffer, and then concentrated to approximately 1 mL and applied to the Sephadex G-100 column (1.6 x 48 cm). The first orange-red colored fractions eluted at a volume corresponding to that expected for BSA; a later small orange band was observed corresponding to the unreacted ferrocene. The fractions of the first band were collected and concentrated to approximately 18 mL. A portion of the protein solution was used for UV-visible and EPR-MS analyses along with the ultrafiltrate blank to determine the extent of ferrocene modification of the BSA. The remaining portion of the modified BSA was washed with deionized water (4 x 50 mL) and concentrated to 1.1 mg BSA/mL. The modified BSA sample was submitted to the hybridoma lab for immunological studies.

Determination of the Extent of HSA Modification

UV-visible Analysis. Native HSA has a single UV-visible absorbance band at 278 nm ($\epsilon = 41000 \text{ M}^{-1} \text{ cm}^{-1}$) due to the tyrosine and tryptophan amino acid aromatic side chains [44]. The electronic absorbance spectrum of the FcHSA modified HSA clearly shows an increase in absorbance to 338 nm (see Figures 3.2 and 3.3). FcHSA has absorbances at 338 nm ($\epsilon = 3000$) and 433 nm ($\epsilon = 100$) [see Figure 3.4]. By using the absorbance at 278 nm to estimate the protein concentration and the absorbance at 338 nm to estimate the ferrocene concentration the extent of modification comes to 14 moles FcHSA/ mole HSA. Since the tyrosine side chain is also susceptible to modification, the concentration of the modified protein determined by UV-visible analysis may be too high (as was later confirmed by HPL-MS).

HPL-MS Analysis. As described earlier in chapter 2 and in more detail elsewhere [45], molecular protein concentrations can be accurately determined based on sulfur content provided the amino acid composition of the protein is known. HSA has 18 cysteines and 4 methionines for a total of 22 moles of sulfur per mole of HSA. The identities of three amino acid residues in HSA are still being debated, but it is thought that one of them is either a glycine or a cysteine residue. Since cysteine is a sulfur bearing

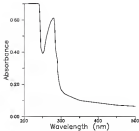


Figure 3.3. UV-Visible Spectrum of Swiss Mouse Serum Albumin (BSA) in 50 mM phosphate buffer, pH 7.0 (1 cm path length).

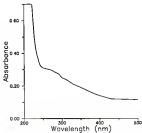


Figure 3.1. Spectra of ferrocenecarboxylic acid modified Serran Serran fibrous in 0.2 M phosphate buffer, pH 7.0 (1 cm path length).

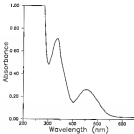


Figure 3-4. UV-Visible spectrum of Paracetamolacetic acid in 50 mM phosphate buffer, pH 7.0 (1 cm path length).

residue and glycine is not a sulfur bearing residue, this introduces an error of 2.5 % into the ICP-AES results in addition to error from the instrument which is reported. By analyzing the Fehrs modified BSA for Fe and S content, the Fe/S ratio obtained showed an average of 33.8 ± 1.0 ferrous groups bound to the BSA (based on 10 moles of S per mole BSA).

Comparison of the UV-visible and ICP-AES results shows that the protein concentration obtained by UV-visible was higher than that obtained by ICP-AES by a factor of 1.4. By using the absorbance obtained at 278 nm and a molar absorptivity of 44000, the protein concentration was determined to be 1.75 μ M, whereas the ICP-AES results based on the sulfur content gave a concentration of 1.25 μ M. By using the ferrous concentration, as determined by UV-visible analysis at 318 nm, and the protein concentration, as determined by ICP-AES analysis, the extent of modification is calculated to be 31.2 ferrous units per BSA, which is more consistent with the ICP-AES results obtained by Fe and S analyses alone. Such increases in the UV absorption of proteins with modified aromatic residues are common. For the standard preparation of tris(trobenzene sulfonate) modified BSA, Good and co-workers not only prepared the modified protein, but also a modified lysine and tyrosine derivative to aid in the quantification of the extent of modification UV-visible spectrometry [27]. Modern

absorptivities for the two amino acid derivatives were obtained and the contribution of the tyrosine modification was estimated and subtracted from the spectrum to obtain a more accurate protein concentration.

Modification of EIA with Folate

In order to facilitate the screening of mouse blood serum for the metabolite specific antibodies, the carrier protein EIA was also modified with Folate via the DCC coupling method above. DCC (3.4 mg) and Folate (3.4 mg) were dissolved in 1 ml of the $\text{NaHCO}_3/\text{DMF}$ buffer. Previously described [Stephens 4-1980] EIA (3.4 mg) was washed with a 50 ml portion of the $\text{NaHCO}_3/\text{DMF}$ buffer and concentrated to approximately 1 ml, and then added to the folic acid solution and allowed to react for 48 hours at room temperature. The reaction was worked up as previously described for the EIA and applied to a Sephadex G-200 column. Two unreacted peaks were evident in the UV chromatograms at the elution values for the EIA. The two light orange fractions were collected and concentrated using the ultrafiltration to a blank for analysis. A portion of the protein solutions were used for UV-visible and ICP-AES analyses, while the remaining solutions were combined, washed with deionized water (4 x 50 ml), concentrated by ultrafiltration to 0.50

ng K124/ml, and submitted to the hybridoma lab for immunological studies.

UV-Visible Analysis. Native K12 has absorption bands at 280 nm ($\epsilon = 1.02 \times 10^5/\text{M}$) of K12p and 340 nm ($0.18 \times 10^5/\text{M}$) of K12f (80). The PEGylated modified K12 shows an increase in absorbance to 280 nm (see Figures 3.3, 3.6 and 3.7). PEGylated has two absorption bands at 218 nm ($\epsilon = 1000$) and 450 ($\epsilon = 100$). (see Figure 3.4). Since the proteins and ferrocene absorbance bands overlap, extent of modification was determined by ICP-AES.

ICP-AES Analysis. K12 Oligos type W-2119, 17.7 mg ferrocene-dried powder was dissolved in 10 ml of deionized water. Based on the analytical data provided by Sigma (94% protein by Biuret test and 4.18% Fe by AA analysis), the actual protein concentration in the volumetric flask was calculated to be 2.580 mg/ml. Based on the copper analysis provided by Sigma the copper concentration in the native K12 solution was calculated to be 1.3 ppm. ICP-AES analysis determined the copper concentration to be $1.25 \text{ ppm} \pm 0.13$. From the sulfur analysis of the native K12 solution, the sulfur concentration was determined to be 17.8 ± 0.7 , which was beyond the linear calibration curve established with standards immediately prior to analysis of the native K12 solution. The sulfur ICP-AES analysis has the slight

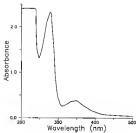


Figure 3.3. UV-Visible spectrum of Native Keyhole Limpet Hemocyanin in distilled water (0.14 mg ml⁻¹, 1 cm path length)

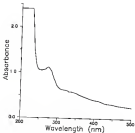


Figure 3.8. UV-Visible spectrum of retroconeylbutyric acid Modified Baylis Elger Reagent (fraction 1) in 50 mM phosphate buffer, pH 7.0 (1 cm path length).

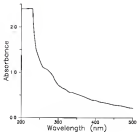


Figure 3.7. UV-Visible spectrum of Perbenzoylmethyl acid modified Kephale lipid Nanocapsule - Fraction 2 in 0.05 M phosphate buffer, pH 7.4 (1 cm path length).

disadvantage of being limited to solutions where the magnesium and calcium concentrations are equal to or less than the sulfur concentration. Calcium and magnesium have spectral lines in the vacuum UV near the sulfur line that interfere in the sulfur analysis and are approximately one hundred times less intense than the S line on a molar basis [88]. Many homocysteines are handled by using 0.1 M CaCl_2 solutions to stabilize the unassociated proteins. Since more data was available for the copper content of ESR, the copper content was used to determine the ESR protein concentration. From the amount of freeze-dried powder, ridge height analysis, molecular weight reported as 808 kDa, and the copper content of the native ESR sample (8 moles of copper per mole ESR or an average of 2.8 moles Cu/mole ESR subunit) can be calculated.

The two P66V6R modified ESR fractions obtained from the Sephadex G-200 gel exclusion column and their ultrafiltration blanks were analyzed for Cu and Fe content via ICP-AES. Based on 2.8 moles Cu/mole ESR subunit, the amount of P66V6R modification was found to be 2.78 and 4.88 ferrocenyl units/subunit ESR for fractions 1 and 2, respectively. Due to the small amount of sample left, the two fractions were combined and washed four times with deionized water to yield a solution of 8.85 μg ESR/mL with an average of 18 moles of P66V6R bound per mole of ESR (2.8 moles ferrocenyl/mole ESR subunit).

CHAPTER 4 EXPERIMENTAL

Introduction

Several organometallic derivatives and organic compounds have been prepared as reagents for the modification of horse heart cytochrome c (HHC), keyhole limpet haemocyanin (KLH), and bovine serum albumin (BSA) and are described in chapters 1 and 3. New halocetyl and haloacrylate alkylating reagents were prepared for the single-site modification of cytochrome c at methionine 43. In addition, organometallic ketocoids were prepared for use as multi-site coupling reagents for the carbodiimide promoted modification of BSA and KLH. Both types of organometallic reagents contain either an iron or a ruthenium metal atom sandwiched between two cyclopentadienyl rings (Cp), with one of the Cp rings being monosubstituted with the corresponding functional group (see Figure 4.1). The organic reagents were prepared as analogues to *p*-bromocetamidophenylferrocene (Pbromofc) [1].

When grouped according to Cp substituent type, monosubstituted metallocene derivatives have ^1H NMR spectra with similar splitting patterns for the Cp protons [1].

For instance, those derivatives with electron withdrawing substituents (such as ketocyclo, para-phenyl derivatives, and acetyl derivatives) have ^1H NMR spectra containing a pair of unsymmetrical A_2B_2 triplets for the substituted cp ($H_{4,6}$ and $H_{5,7}$) downfield from the unsubstituted cp H_1 protons singlet (see Figure 4.1a) [71]. Derivatives with electron donating substituents (such as alkyl derivatives including hexamethyldecylferrocene) generally do not show a chemical shift difference between H_1 and H_4 , although protons on the substituted ring may be shifted to slightly higher field than the unsubstituted cp protons (e.g. bromo-hexamethyldecylferrocene, Foltkshöf, see Figure 4.1b and Table 3.1) [72]. ^{13}C NMR spectra of the monosubstituted derivatives contain a small peak near δ 88-95 for the quaternary cp carbon (C_1), and three resonances between δ 85-95 (consisting of a more intense peak for the unsubstituted cp carbons (C_4), and two smaller peaks for the substituted cp tertiary carbons (C_5 and C_6) upfield from C_1 , except for the ketocyclo derivatives, which have the unsubstituted cp carbon peak, C_4 , between the two substituted cp carbon peaks, C_5 and C_6 , (see Figures 4.3 a and b and Table 4.2).

^1H HETCOR spectroscopy was used to assign some of the ^{13}C NMR resonances (see Figure 4.4a and b). The HETCOR pulse sequence (see Figure 4.5) generates a two-dimensional spectrum where frequency axes are carbon and proton chemical

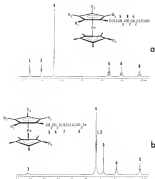


Figure 4.2. Room temperature ^1H NMR spectra of nonsubstituted ferrocene derivatives: a. Toluene, which contains an electron withdrawing substituent; b. Toluene- d_6 , which contains an electron donating substituent (both spectra were referenced to CDCl_3 at δ 7.26).

TABLE 4.1

¹³C NMR Chemical Shifts of Organic and Organometallic Compounds. All spectra were taken in CDCl₃ referenced at δ 7.26. Cp=unsubstituted Cp, Cp'=substituted Cp, and CH₃=Methyl substituent.

Compound	Cp δ	Cp' δ	Cp' δ	CH ₃ δ	Si δ	Si δ	Si δ	Si δ
PCl ₄ Cl	4.24	4.43	4.41	4.43				
[SiH ₅]	4.24	4.44	4.59	4.43				
PCl ₃	4.25	4.44	4.20	4.23				
Ph ₃ SiBr	4.24	4.13	4.13	3.83	2.48	3.41	4.86	
PCl ₃ SiCl	4.24	4.13	4.13	3.88	3.54	3.97	4.92	
PhPhenylSiBr	4.03	4.47	4.28	4.46	2.45	2.81	4.29	
PhPhenyl ₂ (Li ₂)	4.05	4.74	4.47		4.13	4.54		
PhPhenyl ₂	4.03	4.70	4.40		(not given)			
PhPhenyl ₂ Si	4.03	4.54	4.34		2.38	4.45	5.50	
PhPhenyl ₂ SiCl				4.18	2.45	2.15	4.23	2.13
PhPhenyl ₂ SiBr				4.42	2.45	2.14	4.23	2.13
PhPhenyl ₂ SiAc				3.83	2.38	2.13	2.81	2.13
PhPhenyl ₂	4.25	4.43	4.43		3.08	3.70		
PhPhenyl ₂	4.23	4.40	4.37		2.45	2.53		2.04
SiSiBr ₂	4.54	5.04	4.75		2.48	3.42		2.98

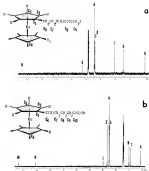


Figure 4.1 Room temperature ^{13}C NMR spectra of a series of ferrocene derivatives. a. TTF b. TTF (Both spectra were referenced to CDCl_3 at $\delta = 40.0$).

Table 4.3

^{13}C NMR chemical shifts of Organic and Organometallic Derivatives. Unless otherwise noted, all spectra were run in CDCl_3 and referenced to CDCl_3 at δ 77.3.

Compound	1	2	3	4	5	6	7	8
							9	10
Potassium	45.1	55.8	57.8	58.8	25.3	185.1		
Potassium	45.5	55.3	57.7	58.8	-0.30	185.8	25.1	25.1
Potassium	45.5	45.5	46.8	75.3	25.8	185.1	137.6 137.8	138.3 137.8
Potassium ₂	81.7	75.8	43.1	75.6			148.1 155.5	151.6 148.1
(LiCl, [80])	45.7	75.3	57.6	59.5			148.3 156.3	156.8 148.3
Potassium ₂	85.8	68.3	83.8	68.8			138.3 155.3	137.3 146.8
Potassium ^a	82.8	73.8	70.8	71.8	185.3	181.6	57.8	55.3
Potassium ^a	75.5	78.7	71.5	72.8	113.8	185.2	81.8 84.1	78.4
Sodium	81.7	73.8	73.8	75.7	101.7	178.7	57.8 15.8	55.8

^a Solvent was CD_3CO and all spectra were referenced at δ 49.6.

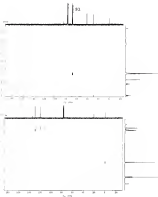


Figure S-4. ¹H NMR spectra of derivatives prepared.
 a. 1a and b. 2a and b.

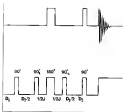


Figure 4.3. The IEEE 802.11b pulse sequence.

shift, peaks appearing in the spectrum correlate a carbon with its neighboring proton(s) [87]. The delay, D_1 , allows the protons to relax to equilibrium a 90 degree pulse is then applied using the decoupler [88]. A delay, $D_2/2$ follows to allow the proton states to evolve [89]. At this point, broad band decoupling of proton dimension is accomplished using the selective proton inversion sequence $90^\circ(x)-1/12-180^\circ(x)-1/12-90^\circ(-x)$, which acts as a 180 degree pulse for those protons not attached to ^{13}C [85]. A 180 degree pulse is applied to ^{13}C at the center of the selective inversion sequence to invert the carbon spin states [89]. A delay $D_2/2$ provides the second period of evolution for the protons [89]. The delays, D_3 and D_4 allow observation of correlation due either to one bond C-H coupling or to long range couplings [89]. After these delays, the free-induction decay (FID) is acquired with proton decoupling [89]. For example, in the NMR spectrum of p-ethoxyaniline, the peak corresponding to the acetylmethylene protons resonance at δ 3.83 also corresponds to the ^{13}C resonance at δ -0.38 indicating that the carbon resonance also corresponds to the acetylmethylene carbon (see figure 4.4b).

Infrared spectra for the derivatives are consistent with reported IR spectra for other monosubstituted anilines derivatives [92]. In general, monosubstituted anilines have IR spectra that contain an absorption band

in the 1000-1100 cm^{-1} region, which metalloenes having both rings substituted each with a bond (see Figure 4.4).

$\text{Cp}^*\text{FeCp}^*\text{R}(\text{R})\text{C}(\text{O})\text{C}_6\text{H}_5$	1348 cm^{-1}	1482 cm^{-1}
$\text{Cp}^*\text{FeCp}^*\text{R}(\text{R})\text{C}(\text{O})\text{C}_6\text{H}_4$	1348 cm^{-1}	1479 cm^{-1}
$\text{Cp}^*\text{FeCp}^*\text{R}_2\text{R}(\text{R})\text{C}(\text{O})\text{C}_6\text{H}_5$	1047 cm^{-1}	1483 cm^{-1}

Electronic spectra of the derivatives prepared are consistent with those of other metalloene derivatives [70]. Typically, the UV-visible spectra of metalloene derivatives contain some bands which are similar to UV-visible absorption bands of their metalloene parent compound. For most of the ferrocene derivatives, the 325 and 440 nm bands of the parent ferrocene moiety are unshifted (see Figure 4.7). The bands at 325 and 440 nm arise from $d-d$ type ligand field transitions [72]. Mass spectral parent ions and C, H, N chemical analyses are consistent with the structures in Figure 4.1.

EXPERIMENTAL PROCEDURES

The preparation and characterization of the ferrocenyl and organic silylating reagents used in chapter 3 are discussed in this section. The ferrocenyl derivatives can be further broken down into two subcategories: those with the functional group directly bound to the ferrocene Cp ring



Figure 4-8. Typical FT-IR spectra of a monosubstituted ferrocene derivative (P600d4).

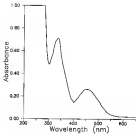


Figure 4.7. Typical UV-Visible spectrum for a mono-substituted ferrocene derivative (FolKbII).

and those with the functional group bound to the para position of phenylferrocene. Ferrocene is considered to be the organometallic analog of benzene based on the similar abilities of the Cp ring and benzene ring to undergo various reactions including: Friedel-Crafts acylation [32] and alkylation [33], demethylation [34], sulfonation [35], nitration (with *n*-butyllithium [36], phenyllithium [37], or sodium azide [38]), arylation with diazonium salts [39], and conversion to *p*-substituted azides [40] by treatment with azobiscyanoates. Consequently, a series of ferrocene alkylating reagents based on a series of phenyl reagents (which are known protein alkylating reagents and have been used to modify methionine residues) were prepared. The phenylferrocenyl derivative was prepared to investigate the effect of the ferrocene moiety's electron donating ability on the reactivity of the alkylating functional group relative to an arylidene electron donating moiety [73], CH_3 , and to hold the haloacetaldehyde functional group rigidly away from the ferrocene iron nucleus.

Subcategory II: the Functional Group Directly Bound to the Ferrocene Cp Ring

Three ferrocenyl alkylating reagents with the reactive functional group directly bound to the ferrocenyl Cp ring were prepared. Iodoacetylferrocene (FalcI) contains a

haloacetyl functional group directly bound to the ferrocene Cp ring and resembles its organic analog, phenacylbromide, which was used by Schrens and Lawton [48] to modify methionine residues in chymotrypsin. The other two derivatives are iodo and bromoacetamidomethylferrocene (Folactol, 81), which contains a haloacetamidomethyl group bound to the ferrocene Cp ring, and resembles their organic analog, *N*-(*N*-phenacyl)methionemethanamide, which was also used by Schrens and Lawton [48] to modify methionine residues in chymotrypsin.

Folactol

Iodoacetylferrocene (Folactol) was prepared from chloroacetylferrocene (Folactol), via halide exchange from chloroacetylferrocene in 88% yield. The halide exchange method used was based on Knaisholtz and Verrier's procedure for the conversion of phenacylbromide to phenacyliodide [49] and the synthesis of Folactol was described by Farrell [51], in a personal communication. One step in Knaisholtz's procedure uses activated charcoal, which ferrocene may adsorb onto; consequently, the activated charcoal step was omitted. Chloroacetylferrocene was prepared by Friedel-Crafts acylation of ferrocene with chloroacetylchloride (see Figure 4-8) [52].



Figure 4.8. Synthesis of *Pds*Et.

To ensure near literature yields [34] of $\text{Fe}(\text{C}_5\text{H}_4\text{Cl})_2$, scrupulously dry columns, chromatographic materials, and gold label aluminumchloride were used. If these dry conditions are not met, little or no chloromethylferrocene is obtained.

NMR Analysis. The chloromethylferrocene has ^1H NMR chemical shifts at δ 4.85, 4.81, 4.83, and 4.76 corresponding to H_1 and H_2 of the substituted Cp, the acetyl methylene protons, and the unsubstituted Cp protons, H_3 , respectively and are in agreement with published values [34]. The iodomethylferrocene produced from halide exchange of the chloromethylferrocene has ^1H NMR chemical shifts at δ 4.85, 4.45, 4.44, and 4.44 (corresponding to the H_1 and H_2 of the substituted Cp ring, the unsubstituted Cp protons, and the iodomethyl methylene protons, respectively). The chemical shift difference observed for the halomethylferrocene methylene protons is in the same direction as that reported for their organic analogues, phenylchloride and

phenacylbromide. The upfield shift of the halogenacyl methylene protons implies that the protons are more shielded and can be explained by electronegativity and heavy atom effects. In going through the halide family from chloride to iodide, the halide becomes less electronegative and thus less able to pull electron density away from the acetyl methylene protons, which results in less deshielding; consequently, the methylene proton resonances are shifted upfield. The heavier halides also have more diffuse outer orbitals so that as the series goes from chloride to iodine/acetylferrocene the iodide will tend to have more diffuse electron-rich orbitals, which shield the acetyl protons and carbon. The halogenacyl methylene protons shift upfield is expected and is in agreement with shifts observed for a series of related compounds.

Elemental Analysis. FeC_8H_8 has absorbance at 338 and 444 nm, while FeC_8H_7 has absorbance at 357 and 473 nm. In general, single acylferrocene derivatives have two weak d-d type absorption bands near 335 and 440 nm and have spectra similar to those of the parent compound, ferrocene [78].

FeC₈H₇Cl₂

Monochloroacetylferrocene ($\text{FeC}_8\text{H}_7\text{Cl}$) was prepared via a multi-step synthesis (see Figure 4.3). Iodoacet-



amidostyrylferrrocene (FOASACF) was prepared via halide exchange from the bromo analog [92]. The conversion procedure was based on the halide exchange used for the preparation of FOACF from FOACBr (see Figure 4.8). In step 1 of the synthesis, ferrrocene (I) was acetoacetylated to form N,N'-diacetylaminostyrylferrrocene (II) according to the procedure of Houser and Lindsey [94]. Compound II was then alkylated by methyl iodide to produce the golden yellow quaternary ammonium salt (III), which can be stored at room temperature indefinitely [94]. The quaternary salt was reacted with potassium cyanide in an exchange reaction to produce the corresponding ferrrocenylacrylate (IV) with triethylamine being evolved [95]. The acrylate (V) is not stable and should be used in a matter of hours (for this synthesis, the nitrile was used within 10 minutes). Compound V was reduced by LiAlH₄ to produce the corresponding primary amine (VI) [94]. While many methods are available for preparing haloacetoaldehydes from alcohols employing haloacetic acids, haloacetylchlorides, or haloaceticanhydrides, the haloacetoaldehydes were reactive toward acylating the amine than alkylating the amine. By running the acylation reaction in a dry non-polar solvent such as hexane, the acylated product will be the predominant product, since the halide will be less likely to dissociate and undergo alkylation. The acylated product (VII) was produced in 42% yield for step 3 of this reaction.

series and is greater than 90% yields for similar compounds described later in this chapter. 2-methoxybenzo-
 ethylferrocene (VII) is a yellow-green air-stable powder with a melting point of 134°C. In step 4, compound VII was converted to the corresponding indenobenzylideneethylferrocene (VIII) via halide exchange, as described earlier for the preparation of Fobaf from FobafH (34). Fobaf is a yellow powder which melts at 141°C. FobafH and Fobaf have been analyzed by mass spectrum, IR, UV-visible, ^1H NMR, ^{13}C NMR, and C, H, and N analyses.

UV-VISIBLE ANALYSIS. FobafH has absorbance at 438nm and 458nm, while Fobaf has absorbance at 438nm and a shoulder at 475nm. Alkylferrocene derivatives typically have UV-visible nearly identical with ferrocene (having absorption bands in the region 330 and 440 nm).

Chemical Analysis. M^+ , parent ion peaks were observed at 143 and 151 m/e for FobafH corresponding to the two nearly equally abundant isotopes of bromine in compound VI. FobafH has an M^+ peak at 187 m/e corresponding to the parent ion peak of FobafH. C, H, and N analyses of both compounds were in agreement with values calculated based on the structures in Figure 4.3.

IR Analysis. IR spectra of the two compounds were taken in chloroform. The spectrum of PMSAOCB contained the following IR frequencies in cm^{-1} : 1680 and 1621, corresponding to the amide N-H stretch; 1620, and 1626, corresponding to aromatic C-H bending modes; 1778 corresponding to a C=O bending mode for the alkyl region; 1661 and 1655 corresponding to C-H bending mode and C=O bending mode respectively; 1620 N-H amide stretching mode for amides; 1477, 1424, 1406, 1329, 873 correspond to the Cp ring breathing and C-H stretching modes associated with a monosubstituted ferrocene derivative [71]. PMSAOCB has a nearly superimposable IR spectrum having the following stretches at 1622 and 1618; a C=O stretch at 1670; and an amide N-H stretch at 1621.

NMR Analysis. ^1H NMR spectrum of PMSAOCB contains a broad singlet at δ 4.55 integrating to one proton for the amide proton; a singlet at δ 4.16 corresponding to five protons; and a singlet at δ 4.13 corresponding to four protons, consistent with a single alkyl substituent on a ferrocene moiety. The two Cp resonances were integrated after using the RESOLF program available on the VNS 180 to enhance the resolution of the two peaks [87]. A singlet at δ 3.33 corresponds to the methyl methylene protons. The doublet of triplet at δ 3.41 corresponds to the methylene protons with a neighboring amide group and is similar to the

methylene group next to an amide group in the spectrum of *trans*-diethylbenzene [11]. The doublet of triplets arises from a doublet splitting from the methylene protons themselves since they are magnetically inequivalent ($J_{\text{H}_1} = 4.8$ Hz; see Figure 4.10) and a triplet splitting from the neighboring methylene protons ($J_{\text{H}_2} = 4.5$ Hz). The doublet of triplets appears at first to be a quartet, however, after using the RESOLV program to enhance the resolution of the spectrum, the shoulders of the overlapping peaks become evident (see Figure 4.12). The triplet at δ 3.54 corresponds to the methylene protons bound to the furanose Cp ring and are coupled to their neighboring methylene protons ($J_{\text{H}_2} = 4.5$ Hz). ^{13}C NMR spectrum of Polakofr contains resonances at δ 35.1, 41.4, 47.8, and 51.8, corresponding to the quaternary Cp carbon, C_q , the substituted Cp carbons, C_{H_1} , and the carbons of the unsubstituted Cp, C_{H_2} . RESOLV 3-D analysis of Polakofr shows that the ^{13}C NMR resonance at δ 47.8 corresponds to the methylene carbon bound to the Cp ring while the resonance at δ 41.4 corresponds to the other methylene carbon. The acetyl and carbonyl resonances occur at δ 19.3 and 165.1, respectively.

Polakofl has a very similar ^1H NMR spectrum to that of Polakofr with the Cp and acetyl resonances unchanged by changing the halide. The amide proton was shifted to δ 4.33 and the acetyl methylene protons appear at δ 3.61. The ^{13}C NMR spectrum of Polakofl is again quite similar to the above



Figure 4.10. Newman projection of methylene carbon of Folate showing the magnetic inequivalence of the two methylene protons.

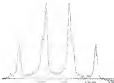


Figure 4.11. The portion of the ^1H NMR spectrum of Polystyrene that contains the doublet of triplets. The *SSOFT* program was used to enhance the spectrum.

derivative, leaving the β and alkyl resonances unchanged, while the acetyl and carbonyl resonances are shifted to δ -0.30 and 188.4, respectively. The increase shielding of the acetyl carbon is typical of carbons bound to iodine and can be explained by the electronegativity and heavy atom arguments presented earlier for the ^1H NMR shift of the acetyl methylene protons upfield upon increasing atomic weight of the halide for PMAI. The acetyl carbon is influenced more than the acetyl proton because it is directly bound to the halide. NMR and core NMR experiments confirm the assignments of the ^{13}C NMR resonances (see Figure 4.12).

Subsequence 2: The Functional Group Bound core to Ferrocene

Since the initial three ferrocenyl alkylating reagents prepared in subsequence 1 did not react with the model compound, EtI , under reaction conditions that would nearly destroy ethylene α , reasons for this apparent lack of reactivity were investigated, with respect to the reactivity of their well-established organic analogues. Ferrocene is an electron donating substituent having a Hammett constant (σ_p) of -0.10 [72]. Could the electron donating ability of the ferrocenyl moiety be contributing electron density to the haloacetyl functional group, making it less electrophilic and thus less reactive? The ferrocenyl

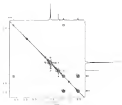


Figure 4.12. COSY 2D spectrum of FolateI.

moiety may have contributed to the apparent lack of reactivity for the iodocarbonylferrocenes; however, the iodoacetamidocarbonylferrocene has two saturated carbon atoms between the ferrocenyl moiety and the iodocarbonyl functional group and thus the electron donation of the ferrocene moiety should not contribute to the latter compound's lack of reactivity. Potential issues that could bring the iodoacetamide group closer to the ferrocenyl moiety allowing the functional group to interact in some way with the ferrocenyl moiety or possibly with other ferrocenylacetamide molecules. Direct comparisons between the organic alkylating reagents and their organic analogues, based on the Hammett constants available, is not valid, since the constants refer to comparisons of substituents versus a phenyl ring; consequently, compounds of the general type $\text{p} = \text{R}_2\text{E}_2\text{N}(\text{R})\text{COOCH}_3$ (where $\text{R} = \text{H}$ or Me and $\text{E} = \text{Cl}$, Br , I) were prepared. The organic substituent having a Hammett constant (σ_p) closest to that of the ferrocenyl substituent is a methyl group ($\sigma_p = +0.17$) [23]. The phenyl spacer group offers not only the ability to compare the electron donating substituents influence on the rate of alkylation, but serves to rigidly hold the halocarbonyl functional group away from the ferrocenyl moiety. The synthesis and characterization of the phenyl spaced series of organic and organometallic compounds will be discussed in the next section.

Organic Analogs

p-chloro and bromocetanilide were prepared by reacting *p*-toluidine with the appropriate anhydride in a 1:1 ratio in benzene at room temperature (see Figure 4.13). Both bromo and iodocetanilide were obtained in greater than 80% yield. *p*-Iodocetanilide was prepared from the corresponding chloro derivative (by halide exchange as described earlier for *p*-tol) in greater than 80% yield (see Figure 4.13).

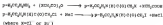


Figure 4.13. Synthesis of organic analogues.

¹H NMR spectra of the three derivatives show a shift of the acetyl methylene proton singlet upfield as the halide goes from Cl⁻ to Br⁻ to I⁻, indicating increased shielding of the acetyl methylene protons, which is consistent with the trend seen for the compounds in subcategory 1 (see Table 4.1). A similar trend is seen for the acetyl methylene carbon for this series of organic compounds (see Table 4.1). *p*-Bromocetanilide was used by Lehman and Lannon [48] to modify methionine residues in thyrocytopsin. *p*-Bromo-

modify methionine residues in thyrotyrosin. *p*-bromo-
acetamidophenylferrocene (Polthensulfur) was prepared as the
organosulfur analog to bromoacetamide.

Polthensulfur

p-bromoacetamidophenylferrocene was prepared via a four
step synthesis (see Figure 4.14). In step 1 *p*-bromoniline
was diazotized at 0°C in concentrated HCl to produce the
corresponding diazoniumchloride by following the procedure
of Meerwein (substituting HCl for H_2SO_4) (18).



Figure 4.14. The synthesis of Polthensulfur.

Special care was taken to ensure that the reaction
temperature stayed between 0-5°C upon addition of the sodium
nitrite solution. The diazonium salt was then coupled with
ferrocene in glacial acetic acid at room temperature to

produce dark purple crystals of p-nitrophenylferrocene (Förster₉) [180]. ¹H NMR chemical shifts [71], ¹³C NMR chemical shifts [201], and melting point [188] of the Föster₉ prepared were in agreement with reported values. In step 3 p-nitrophenylferrocene was reduced under 50 psi H₂ catalyzed by PtO₂ at room temperature to produce a golden yellow solution of p-aminophenylferrocene (Föster₁₀) [181]. Melting point of the yellow powder was in agreement with literature values for Föster₁₀ [182]. ¹H NMR and ¹³C NMR spectra of Föster₁₀ contain the same ferrocenyl and phenyl features of the parent compound (shifted slightly) and a broad singlet at δ 3.4, which integrates to 2 protons as would be expected for the amine derivative (see Table 4.1). In step 4 the aminophenylferrocene is acylated by [ReCl₅OEt]₂, similar to the reactions used for the earlier bisacetamide derivatives, to produce the p-bromo acetamidophenylferrocene in 50% yield melting at 150 °C [183.]; However, this particular reaction was run at room temperature. Earlier attempts at preparing the bis-acetamidophenylferrocene at refluxing benzene temperatures yielded a mixture of products, which, when chromatographed, yielded a mixture of ferrocene and disubstituted phenylferrocenes.

Chemical Analysis. Mass spectral analysis of Föster₁₀ gave parent ion, M⁺, peaks at m/e 157 and 170,

which correspond to the two nearly equally abundant isotopes of bromine in *Perfluorobenzene*. ^1H , ^{13}C , and ^1H chemical analysis of *Perfluorobenzene* was in agreement with calculated values.

NMR Analysis. ^1H NMR spectrum of *Perfluorobenzene* in CDCl_3 contains the wide broad singlet at δ 8.1; the phenyl set of doublets at δ 7.6 and 7.8; the unsymmetrical ferrocenyl triplets at δ 4.6 and 4.8; the acetyl methylene protons at δ 4.64 and the unsubstituted Cp protons singlet at δ 4.23 (see Figure 4.15). The chemical shift difference for the phenyl protons is remarkably small with respect to the two precursor phenylferrocene derivatives prepared (see Figure 4.14). ^{13}C NMR spectrum of *Perfluorobenzene*, taken in CDCl_3 , contains a phenyl region similar to *Perfluorobenzene*, a ferrocenyl region typical of unsubstituted ferrocene derivatives, and an acetyl carbon at δ 19.8 similar to the other two bromobenzene compounds prepared. Similarity between *Perfluorobenzene* and *Perfluorobenzene* can be seen in the ^1H and ^{13}C NMR spectra of the two compounds. The ^1H NMR spectra of the two compounds have the same chemical shifts for the wide and acetyl protons indicating a similar electronic environment for the ferrocenyl functional group in these derivatives. The phenyl region of the two ^1H NMR spectra are slightly different in that the downfield doublets are at the same chemical shift for the two compounds, but the upfield set of phenyl doublets for *Perfluorobenzene* are δ 0.4

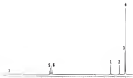


Figure 4.15. Room temperature ¹H NMR spectrum of Porphobilinogen in CD₃OD (referenced to CD₃OD at δ 3.36).

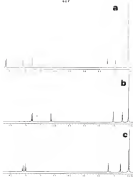


Figure 4.14. ^{13}C NMR spectra of the phenylferrocene derivatives prepared. a. $\text{Pb}(\text{OAc})_2$, b. $\text{Pb}(\text{PHOS})_2$, c. $\text{Pb}(\text{PHOS})_2$.

upfield from that of Porphthalaldehyde. The small chemical shift difference between the two phenyl resonances in Porphthalaldehyde may be due to the greater extent of delocalization of ferrocene and phenyl electrons through their connecting π systems. Such extensive delocalization even in the ferrocene-phenyl derivative is unavailable for the acetyl-phenyl derivative in the organic analog. The chemical shift difference for the Porphthalaldehyde phenyl region is even smaller than that of the two ferrocene precursor compounds (see Table 4.1). The ^{13}C for phenyl and carbonyl resonances for the two derivatives are within ± 2 of each other. The acetyl carbon resonances are within ± 8.2 of each other, also indicating a similar electronic environment for the functional group for the two derivatives.

Ultraviolet and IR Analysis. The electronic absorption spectrum of Porphthalaldehyde contains several absorption bands at 441, 349, 316, 290, and 258 nm. Ferrocenylphenyl derivatives typically have 4-6 absorption bands. The two bands between 290-350 nm are intense and come from the phenyl substituents and metal-ligand interactions; the two bands around 310 and 440 nm are less intense and correspond to π - π^* transitions in the ferrocene moiety. The organic analog had a single intense π - π^* absorption band at 252 nm.

FTIR spectra of the two bromoacetamidophenyl derivatives were taken in chloroform, giving spectra that were nearly identical to the spectrum obtained and discussed for Fekahelir. The carbonyl stretching frequencies for the organic and organometallic compounds were both 1685 cm^{-1} .

Enkephalins Enkeimide

Three metalloene ketonimide were prepared for the multi-site saturation of nociceptin nociceptin (NNA) and tachykinin ligand nociceptin (NNA) as discussed in chapter 2. The ketonimide (general type $\text{C}_6\text{H}_5\text{C}(\text{O})\text{C}(\text{O})\text{M}_x\text{COOH}$ where $\text{M} = \text{Fe}$ or Mn and $x = 2$ or 3) were prepared in high yield via Friedel-Crafts acylation of the metalloene by using the corresponding anhydride and gold label aluminum chloride catalyst and following the procedure of Rhinshart and co-workers (see Figure 4.17) [46].



where $x = 2$ or 3 .

Figure 4.17. Synthesis of the metalloenyl ketonimide.

The only exception to Shinerhart's procedure was in the work up. Instead of adding concentrated acid directly to the BaSO_4 solution, the solution was lyophilized to remove the water, then redissolved the powder in absolute methanol. The ferrocene derivative dissolved, leaving the sodium tetrakisulfate undissolved. The BaSO_4 was filtered off and the remaining methanolic solution acidified with 2 M HCl to precipitate the acetolactoseryl ketoside. Melting points of the two ferrocene derivatives were in agreement with published values [80]. ^1H NMR spectra for the three derivatives were similar, with each spectrum containing a pair of unsymmetrical triplets and a singlet for the substituted and unsubstituted Cp protons and a set of triplets and in the case of the butyric derivatives a quintet for the substituent protons (see Table 4.1 and Figure 4.2). The substituted and unsubstituted Cp protons of the ruthenoseryl butyric acid derivative were approximately 0.2 ppm downfield from the corresponding ferrocene protons, while the two substituent triplets are 24.16 upfield from the corresponding ferrocene derivative. Interestingly, the ruthenosene substituted quintet is 0.4 ppm downfield from the corresponding ferroceneylbutyric acid quintet. Coupling constants for the three derivatives are nearly identical. UV-visible spectra were recorded for the two ferrocene derivatives. FERROS has a λ_{max} at 337 nm.

($\epsilon = 1800$) and 450 nm ($\epsilon = 232$). ^{13}C NMR spectra of the three derivatives were quite similar (see Figure 4.4b).

Experimental Section

All NMR spectra were run on a Varian VXR 300 MHz operating at 300.625 MHz for proton and 75.424 MHz for carbon-13. Chloroform- d_3 , acetone- d_6 , and water- d_2 were purchased from Merck, Sharp, and Gohm. The chloroform was dried over molecular sieves to remove water that appears at δ 3.3. Acetone was not dried and had a water peak at δ 2.5. C, H, N analyses were performed at the university facilities. IR spectra were run on Nicolet FT-18. Melting Points were determined on a Hoover melting point apparatus. Mass Spectral data was run on a Nicolet FT-18 except for Tetrahydrofuran which was run on a Finnigan 40 at the university facilities. Unless otherwise noted, all references for experimental procedures contain a complete description of the experimental steps needed to prepare the compounds.

Preparation of $\text{Cp}^*\text{Fe}(\text{Cp})\text{SiRICH}_2\text{L}$. (see Figures 4.4 for reaction scheme) $\text{Cp}^*\text{FeCp}(\text{O})\text{CH}_2\text{Cl}$ (P05AC1; 180 mg) was prepared according to literature procedure of Nakaguchi by using scrupulously dry MgCl_2 and atmosphere AlEt_3 (quid label, Aldrich) in 2.66 yield (70%). The purified $\text{Cp}^*\text{FeCp}(\text{O})\text{CH}_2\text{Cl}$

was then converted into the corresponding isocyanatylferrocene (FcNCO) via exchange reaction in 34% yield. FcNCO (180 mg) was dissolved in 1.5 ml of dry acetone. NaI saturated dry acetone (3 ml) was added to the isocyanatylferrocene solution and the exchange reaction was allowed to proceed overnight. The resulting NaCl precipitated out and was filtered off. The acetone was evaporated off leaving a red oil that was dissolved in benzene and washed with deionized water to remove any remaining sodiumiodide. The benzene solution was dried over MgSO_4 overnight. The crude isocyanatylferrocene was chromatographed on an alumina in a pipet using petroleum ether and benzene. ^1H NMR (CDCl_3) shifts are relative to SiMe_4 at 47.26, 44.45 (t, 8.0 H, $J=1.98$ Hz); 4.48 (t, 8.0 H, $J=1.98$ Hz); 4.14 (s, 4.0 H); 4.14 (s, 8.0H).

Preparation of $\text{Li}(\text{FcOCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3\text{COOCH}_2\text{CH}_2\text{Br})$. (see Figure 4.9 for reaction scheme) Ferrocene (38.8 g) was aminomethylated by 5.5 ml of 8,8,8',8'-tetrakis(methylamino)octane producing 7.8 ml of dimethylaminomethylferrocene (step 1, 21% yield) [74]. The dimethylaminomethylferrocene was then acylated by 7.8 ml of methyl iodide to produce 8.0 g (step 2, 21% yield) of the corresponding quaternary salt [74]. Steps 1 (yield 21%) and 2 (yield 21%) were repeated to produce an additional 15 grams of the quaternary salt. The quaternary ammonium salt (38.8 g) was then converted to the

corresponding nitrile (11.1 g), via exchange reaction with 10.9 g of NH_4SCN (step K) [16]. The nitrile was hydrogenated within thirty minutes to the corresponding amine by 45 ml of 1 M LiAlH_4 (step L; 81% yield) [14]. α -Aminoethylferrocene (2.18 mEq 4.1-0.155 g/ml) was dissolved in 25 ml of benzene in a 100 ml three-necked round bottom flask equipped with a nitrogen inlet and condenser. Bromoacetic anhydride (2.8 g) was added to the ferrocene solution. The reaction mixture was heated until the benzene began refluxing and then was allowed to reflux for 40 minutes. After the reaction had cooled to room temperature, the solution was poured into 100 ml of deionized water. The benzene solution was extracted and dried over MgSO_4 . The benzene was removed and the residue was recrystallized from cyclohexane yielding yellow-green crystals of bromoacetic acid ethylferrocene (Polaroid® yield 42%). MP 125-126°. n_D^{20} 1.49,553 m/s. ^1H NMR (CDCl_3) 4.58 [broad s, 0.5 H]; 4.15 (s, 4.8H); 3.44 (s, 3.3H); 3.41 (dt, 3.8H, $J=6.5$ Hz and $J=6.9$ Hz); 2.68 (t, 1.5H, $J=6.5$ Hz). ^{13}C NMR (CDCl_3) δ = 145.1; 95.1; 81.8; 81.4; 82.1; 81.4; 78.4; and 25.1. λ_{max} at 418 nm and 124 nm. Analytical: Calculated for $\text{C}_{12}\text{H}_{15}\text{FeOBr}$: C, 48.04; H, 4.41; Br, 4.54. Found: C, 47.87; H, 4.40; Br, 3.58. FTIR (CDCl_3): 2985; 2921; 2420; 1600; 1578; 1451; 1446; 1320; 1277; 1204; 1046; 933; 877 cm^{-1} .

Preparation of $\text{C}_6\text{H}_5\text{C}(\text{CH}_3)_2\text{CH}_2\text{CH}(\text{CH}_3)\text{COOCH}_2\text{CH}_3$. (see Figure 4.1 for reaction scheme) Potassium (0.5 g) was dissolved in 2.5 ml of dry acetone (molecular sieve). A saturated solution of NaI in dry acetone (2.5 ml) was added to the ferrous solution; the exchange reaction was allowed to proceed overnight. NaI was filtered off, 5 ml of benzene and 5 ml of deionized H_2O were added to the remaining solution. The benzene layer was extracted and dried over MgSO_4 . The dried ferrous solution was applied to an alumina column (200g alumina neutral, unactivated). Potassium was eluted with 1:1 benzene and petroleum ether (medium boiling range). A 70% yield of potassium as a yellow powder was obtained for the conversion reaction. MP 114 $^\circ\text{C}$. NMR 10 τ . ^1H NMR (CDCl_3) shifts are relative to CHCl_3 at 77.25. δ : 4.22 (broad s, 2.5 H); 4.10 (s, 2.0H); 3.44 (s, 1.4H); 3.37 (dt, 2.2H, $J=6.5$ Hz and $J=8.8$ Hz); 3.25 (t, 1.4H, $J=6.5$ Hz). ^{13}C NMR (CDCl_3) shifts are relative to CHCl_3 at 77.0. δ : 164.4; 65.4; 62.1; 61.3; 47.7; 29.3; 26.3; and -0.361. Analytical (calculated): C, 62.10; H, 4.96; O, 3.33. Found: C, 60.42; H, 4.88; O, 3.34. FTIR (CHCl_3): 3009; 3026; 3036; 3059; 2934; 2941; 2979; 2983; 1477; 1426; 1086; 838; 677.

Attempted synthesis of $\text{C}_6\text{H}_5\text{C}(\text{CH}_3)_2\text{CH}(\text{CH}_3)\text{CO}_2\text{H}$. (see Figure 4.10) Perfluorooctanoic acid (2.5 g) was converted by reaction with sodium azide in a basic solution to the corresponding azide (100). An attempt at converting the

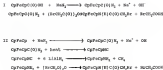


Figure 4.18. Reaction schemes for the synthesis of $\text{OpFeOp}(\text{CO})\text{CH}(\text{O})\text{CH}_2\text{Br}$. (I) The route attempted and (II) the route suggested as an alternative.

acids directly to the corresponding bromoesteramide, which was based on the procedure for converting the acids to the corresponding esteramide [184], failed to produce the desired product. The red acids oil was added to one equivalent of bromoacetylbenzoylchloride in 10 ml of toluene. The reaction was heated to 80°C and slow evolution of H_2 was observed. A yellow precipitate formed and within two minutes decomposed into a black tar. Future attempts at preparing the bromoesteramideferrocene derivative should proceed by a modified Curtius reaction to produce the nitrile [185], reduction to the corresponding amine [186], and acylation with bromoacetylbenzoylchloride by using the procedure employed for conversion of aminoethylferrocene to the corresponding bromoesteramidoethylferrocene (see the alternative pathway in Figure 4.18).

Preparation of $C_6H_5CH_2CH_2CH_2CH_2COOH$. (see Figure 4.17)

Orange crystals of ferrocenophenylacetic acid were prepared by Friedel-Crafts acylation of 14.5 g of ferrocene with 5.0 g of glutaric anhydride and 15.1 g of $AlCl_3$ following the procedure of Birchbark [45] with the following deviations to the work up procedure. After extraction of the ferrocene acid into saturated sodium carbonate solution, the ether was removed and the ferrocene solution was lyophilized to dryness. A portion of the lyophilized powder was converted into the acid. Excess Na_2CO_3 was precipitated out with

washed and filtered off, the resulting methanolic solution was acidified with 4M HCl yielding an orange precipitate having a mp corresponding to the literature value (118-119°C, dec.) [45]. ¹H NMR (CDCl₃) shifts are relative to CDCl₃ at δ 7.35: 4.4.48 (t, 2.0 H, J=1.3 Hz); 4.38 (t, 2.0 H, J=1.7 Hz); 4.35(a, 4.4 H); 4.41 (t, 2.0 H, J=1.3 Hz); 3.58 (s, 2.0 H, J=7.1 Hz); 3.46 (quintet, 2.0 H, J=7.1 Hz). ¹³C NMR (CD₃CO) referenced to CD₃CO at 48.6 : 117.47 (48.5); 79.8; 74.7; 71.4; 61.8; 59.4; 54.1. UV-visible λ_{max} (cyclohex, 200%) at 328 nm (ϵ = 7400) and 326 nm (ϵ = 332).

Preparation of CaFeCyclopentadiene-5,6-dione. (see Figure 4.17).

Yellow crystals of ferrocenecyclopentadiene acid were prepared following the procedure of Miescher [28], with the same exceptions noted for the ferrocenecyclopentadiene acid above.

Ferrocene (34.8 g) was acetylated by 4.8 g of succinic anhydride producing ferrocenecyclopentadiene acid. mp 167°C corresponds to the reported value (166-167.5°C) [46]. ¹H NMR (CDCl₃) shifts are relative to CDCl₃ at δ 7.35: δ 4.48 (s, 1.8 H, J=1.3 Hz); 4.35 (t, 2.0 H, J=1.6 Hz); 4.34 (s, 2 H); 3.18 (s, 1.1 H, J=1.5 Hz); 3.76 (s, 1.5 H, J=1.6 Hz). ¹³C NMR (CD₃CO) referenced to CD₃CO at δ 48.6: 105.47 (105.4); 61.4; 71.4; 71.4; 74.8; 57.8; 59.1. UV-visible λ_{max} (phos 100%, pH=7.1) at 285.4 nm (ϵ = 13,34).

Preparation of $\text{Cp}^*\text{Fe}(\text{C}_6\text{H}_5\text{COO})_2\text{CH}_3\text{COOH}$. (see Figure 4.17) Light yellow crystals of ruthenocenylacetic acid were prepared following the procedure for the corresponding ferrocene derivative above. Ruthenocene (1.8 g) was acylated by 0.85 g of acetic anhydride to produce ruthenocenylacetic acid. IR KBr (CHCl_3) shifts are relative to CHCl_3 at 47.85; δ : 5.88 (t, 2.2 H, $J=1.82$ Hz); 4.75 (t, 2.4 H, $J=1.82$ Hz); 4.56 (s, 3.6 H); 3.88 (t, 2.1 H, $J=9.15$); 3.48 (h, 2.2 H, $J=9.65$ Hz); 3.35 (quartet, 2.1 H, $J=9.65$ Hz). ^{13}C NMR (CDCl_3) shifts are relative to CDCl_3 at δ 77.0: 161.75; 178.72; 85.48; 75.00; 75.00; 76.87; 87.88; 88.02; 18.82.

Preparation of $p\text{-NO}_2\text{C}_6\text{H}_4\text{-Cp}_2\text{Fe}(\text{C}_6\text{H}_5\text{COO})_2\text{CH}_3$. (see Figure 4.18 for reaction scheme) p -nitrophenylferrocene (FePhenNO_2) was prepared by diazotizing 18.7 g of p -nitroaniline with 14.8 g of NaNO_2 in 180 ml of concentrated HCl at 0°C [58] and reacting the diazonium chloride salt with 18.0 g of ferrocene under nitrogen at room temperature [180]. The crude mixture was chromatographed on 150 g of deactivated neutral alumina. The purple band corresponding to the monophenylated ferrocene was eluted with 1:1 petroleum ether (BP 30-60°C) and benzene. The fractions containing pure FePhenNO_2 were pooled. BP 143-145°C 1.16 (140°C) [180]. IR KBr (CHCl_3) shifts are relative to CHCl_3 at 77.25 and corresponded to the values reported by Rousillon [75].

δ : 8.5 (d, 2.0 H, J=8.4 Hz); 4.8 (d, 1.0 H, J=8.4 Hz); 4.74 (t, 1.4 H, J=1.47 Hz); 4.42 (t, 1.2 H, J=1.45 Hz); 4.05 (m, 8.0 H). ^{13}C NMR (CDCl_3) shifts are relative to CDCl_3 at δ 77.0 corresponds to the values reported by Tsai [14]: 148.1, 138.8, 81.7, 70.8, 70.8, 67.3. Following the

procedure of Lohse, et. al, 1.3037 g of p-nitrophenylisocyanate was reduced to the corresponding amine at room temperature using 3 g of FeO , catalyst under 83 psi H_2 in a pressure apparatus [18]. The hydrogenation produced 418 mg (yield 84%) of yellow powder (FePhenNH_2) melting at 104°C (lit. 104-108)(19), with ^1H NMR (CDCl_3) relative to CDCl_3 at δ 7.28 (t, 2.16 H, J=8.4 Hz); 6.85 (d, 1.4 H, J=1.4 Hz); 4.54 (t, 1.4 H, J=1.4 Hz); 4.24 (t, 3.2 H, J=1.4 Hz); 4.01 (m, 8 H); 3.5 (broad m, 2.0 H). ^{13}C NMR (CDCl_3) shifts relative to CDCl_3 at δ 77.0. δ :

148.8, 127.1, 118.3, 84.4, 69.4, 68.2, 68.4. p- $\text{NH}_2\text{C}_6\text{H}_4\text{OPeOp}$ (170 mg) was dissolved in 5 mL of benzene. Fromosulfide anhydride (181.8 mg) was dissolved in 1 mL of benzene and then added to the foregoing solution. Within 3 minutes a golden yellow precipitate was observed; the reaction proceeded for 30 minutes. The golden yellow powder (28.8 mg) was collected, the remaining solution was washed with deionized water. The benzene layer was removed and dried over Ba_2P_2 (yield 84%). MP 100°C (Dec.) IR: 1610, 1560. ^1H NMR (acetone- d_6) shifts are relative to $\text{CD}_3\text{OD}/\text{CD}_3\text{S}$ at δ 3.88. δ : 9.52 (broad s, 0.4 H); 7.54 (d, 2.1 H, J=6.75 Hz);

4.88 (s, 1.5 H, J=1.88 Hz); 4.28 (s, 1.5 H, J=3.88 Hz); 4.00 (s, 4.7); 4.00 (s, 3.3). ^1H NMR (CDCl_3) relative to CDCl_3 , δ 71.2. δ 128.12, 128.0, 134.3, 127.3, 128.3, 70.18, 68.7, 47.0. Analytical Calculated: C, 54.31; H, 4.58; N, 3.82. Found: C, 51.74; H, 3.88; N, 3.18. FTIR (CDCl_3): 3485; 3031; 3000; 1778; 1431; 1481; 1401; 1329; 1323; 1277; 1223; 1047; 828; 877. IR-visibls (CH_2Cl_2): 838; 443; 348; 298; 258 nm.

Preparation of $\text{mH}_2\text{C}_6\text{O}_4\text{E}_2\text{NIRICOOCH}_2\text{CH}_2\text{I}$. (see Figure 4-14). *p*-Toluidine (4.86 g) and chlorosulfonic anhydride (5.97 g) were weighed out and placed into a 250 mL round bottom flask equipped with a stirring bar. Sulfuric (180 mL) was added and a condenser was fitted onto the flask. The temperature was raised to produce refluxing benzene. Upon refluxing, the reaction was allowed to proceed for 10 minutes. The heating mantle was removed and the reaction flask was allowed to cool to room temperature. The solution was then poured into a flask containing 100 mL of deionized water. A white-yellow sticky precipitate was collected and recrystallized with ethanol and water to yield sticky white crystals that melt sharply at 148.8 (lit. 145-149)(107). Yield 89.94%. Analytical Calculated: C, 58.87; H, 5.89; N, 7.62. Found: C, 57.84; H, 5.90; N, 7.54. ^1H NMR (CDCl_3) relative to CHCl_3 , δ 7.18. δ : 4.2 (broad s, 1.5 H); 7.81 (d, 2.5 H, J=8.4 Hz); 7.15 (s, 1.1 H, J=6.1 Hz); 4.14 (s, 1.5 H); 3.12 (s, 3

mp. $^{\circ}\text{C}$ NMR (CDCl_3) relative to CHCl_3 at δ 77.8: 188.8, 135.69, 124.14, 123.49, 123.28, 81.82, 38.76.

Preparation of $p\text{-H}_2\text{CC}_6\text{H}_4\text{N}(\text{CH}_2\text{CH}_2\text{CH}_2)_2$. (see Figure 4.13).

$p\text{-H}_2\text{CC}_6\text{H}_4\text{N}(\text{CH}_2\text{CH}_2\text{CH}_2)_2\text{Cl}$ (8.8 g) was converted into the corresponding p -iodoacetanilide via exchange reaction with NaI in dry acetone as described earlier for the conversion of Toluidine to Toluidol. BP 162.8 (Lit. 161-163) (147). ^1H NMR (CDCl_3) shifts relative to CHCl_3 at δ 7.29. 7.43 (overl. m, 4.8 H); 7.18 (d, 1.5 H, $J=8.4$); 7.12 (d, 2.1, $J=8.3$ Hz); 3.83 (q, 2.1 H); 2.16 (s, 3 H). ^{13}C NMR (CDCl_3) shifts are relative to CHCl_3 at δ 77.8. δ 188.8, 134.7, 124.7, 123.4, 123.3, 38.76, 8.08. Analytical Calculated: C, 59.29; H, 5.49; N, 3.22. Found: C, 59.29; H, 5.41; N, 3.08. Yield 73%.

Preparation of $p\text{-H}_2\text{CC}_6\text{H}_4\text{N}(\text{CH}_2\text{CH}_2\text{CH}_2)_2\text{Br}$. (see Figure 4.13).

p -Toluidine (423 mg) was dissolved in 4 ml of benzene with stirring. 1.031 g of bromoacetic anhydride was dissolved in 1 ml of benzene then added to the toluidine solution. Two minutes after the addition of the anhydride, a white precipitate began forming. The reaction was allowed to proceed for 50 minutes. The fleshy white needles were recrystallized from acetone and H_2O . BP 161.5-162.5. Analytical Calculated: C, 47.39; H, 4.45; N, 6.16. Found: C, 46.84; H, 4.34; N, 6.07. ^1H NMR (CDCl_3) shifts relative

to CHCl_3 at δ 7.25. 8.10 (broad s, 1.0 H); 7.81 (d, 1.0 H, $J=8.1$ Hz); 7.16 (d, 1.0 H, $J=8.1$ Hz); 6.82 (s, 2.0 H); 1.00 (s, 3.1 H). ^1H NMR (acetone- d_6) relative to $\text{CH}_3\text{COOCH}_3$ at δ 2.01. 7.45 (broad s, 0.9 H); 7.50 (d, 1.0 H, $J=8.45$ Hz); 7.16 (d, 1.0 H, $J=8.3$ Hz); 3.96 (s, 3.1 H); 2.14 (s, 3.1 H). ^{13}C NMR (CDCl_3) relative to CHCl_3 at δ 77.4: 165.2, 155.9, 154.71, 150.48, 129.14, 128.52, 128.21. FTIR (CHCl_3) = 1626; 1621; 1620; 1617; 1481; 1480; 1480; 1477; 1429; 1068; 928; 877. UV-vis: λ_{max} (CH_2Cl_2): 263 nm ($\epsilon = 18,200$).

CHAPTER 4 CONCLUSIONS

In Chapter 3, the overall conclusions of the research described in the preceding three chapters are presented. Two types of new ferrocenyl alkylating reagents were prepared for the single-site modification of cytochrome c. The first type has the alkylating functional group directly bound to the ferrocene π ligand, whereas the second type has the functional group bound in the para position of the phenyl ring in phenylferrocene. Reagents of the first type failed to alkylate the model compound, ethylnitrophenoxide (EtNO₂), even though their organic analogues did alkylate EtNO₂ under less stringent reaction conditions. To investigate the factors that might be influencing the alkylating ability of the ferrocenyl reagents *p*-bromodinitrophenylferrocene (PbFenNO₂) was synthesized. PbFenNO₂ offers two advantages over reagents of the first type, where the functional group is held rigidly away from the ferrocene group and the electron-donating ability of the ferrocene group can be compared to that of organic substituents on the organic analogues. PbFenNO₂ did alkylate EtNO₂. The reaction of the ferrocene derivative

with HbHb was two orders of magnitude slower than the reaction of its organic analog with HbHb. No direct electronic comparison can be made between the two types of organometallic alkylating derivatives. Attempts to modify cytochrome c with PPhenylstannide did not yield a modified protein, unfortunately, PPhenylstannide is quite water insoluble. An attempt to modify PPhenylstannide was made and the resulting water-soluble compound was used to modify cytochrome c; however, no modified protein was obtained. A logical extension of this work would be to exchange the bromide in PPhenylstannide for triflate, tosylate, or iodide all of which are better leaving groups than bromide and to incorporate solubilizing groups into the ferrocene derivative to increase its water solubility. Electron withdrawing and electron donating substituents could be incorporated into the ferrocene moiety to investigate the influence of the electron donating ability of the ferrocene group on the alkylating ability of the methanide functional group.

As mentioned in detail in chapter 2, the Tied and others have modified the His 13 residue of cytochrome c with several triphenyl derivatives (24, 25). In a recent study they found evidence that electron transfer in the cytochrome c system is gated (26). Our current theory about electron transfer reactions is based on interactions between small complexes and more recently between small complexes and

proteins. As we go to more complex systems and modes of interaction, our theories must be reevaluated. Isted's observations offer us a new challenge toward understanding electron transfer in biological systems. Building another system such as the ferrocenecytochrome *c* system to verify these results with the added advantages of modifying an amino acid which lies in the area of the protein implicated as the site of physiological electron transfer between cytochrome *c* and its biological redox partners may help to increase our knowledge of protein-metal molecule interactions. In addition, the ferrocene system permits varying the driving force of the electron transfer between ferrocene and the heme iron without making major changes in the first coordination sphere of the metal center.

In a second project, the multi-site modification of the carrier proteins, bovine serum albumin (BSA) and lysozyme (Lys) hemoprotein (LH), was undertaken to develop antibodies that are specific for the metalloproteins. Such antibodies may find use as radiotracers and analytical reagents. Antibodies against the hemoLH complex of Indium have been developed by Hsuan and co-workers and are being developed as imaging agents. Metalloproteins offer advantages over traditional transition-metal complexes by being neutral compounds and generally, non-toxic. Breslow and co-workers have modified cytochrome *c* with ferrocene derivatives for conjugation as synthetic enzymes. The

ferrocene synthetic enzyme demonstrates the highest rate enhancement as far achieved by a synthetic enzyme and has high stereospecificity. Results obtained from immunological studies indicate that the ferrocene modified proteins are not eliciting a specific immune response. Although others have regarded ferrocene as having a strong antigenic effect, the derivative used in this study seems to suppress the immune response even to the carrier proteins. The results seem to indicate that either the ferrocene modified proteins are quickly eliminated from the mouse blood serum or that the ferrocene blocks the immune system's recognition of the carrier protein. A logical extension of this work should include modification of the carrier proteins by ferrocene derivatives employed by Flacker and co-workers (discussed in chapter 1) since they have been demonstrated to be good haptens. In addition, ionizable groups such as nitroates should be incorporated into the ferrocene derivative to create an area in the derivative with a hydrophobic and a hydrophilic surface which seems to increase the likelihood of antibody formation.

For both the multi-site and single-site modification projects, an analytical method was developed to determine the extent of protein modification even if the native protein (for example BSA) was not a metalloprotein. The method relies on the use of the sulfur oxidation line in the vacuum ultraviolet for determination of the sulfur content.

of the sample. The concentration of the protein can be determined based on sulfur content of the sample provided the number of methionine and cysteine residues in the protein are known. The method described in more detail in chapter 1, offers the advantage of determining both metal and protein concentration by using the same method, in addition, the method is simple and requires little sample preparation.

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BIOGRAPHICAL SKETCH

Cynthia Bernadette Walton was born on February 23, 1941 to Richard and Shirley Walton of London Grove, Pennsylvania. Cindy grew up in London Grove, where her grandparents have a farm that has been in continuous operation for over 100 years. Her parents own and operate a commercial rose business that her great-grandfather started over 50 years ago. She is the eldest of the Walton's four children and is followed by Richard Thomas, Frederick Pacey, and Linda Sue Walton.

Cindy graduated from Unionville High School in Unionville, Pennsylvania in 1959. Cindy received her B.S. in chemistry from The Pennsylvania State University in 1963, where she did undergraduate research with Dr. Bernard Hickey and Dr. Maurice Shuman. In Penn State, she was in the chemistry honor society, taught freshman chemistry lab, and was on the Dean's list. After receiving her B.S., Cindy began her doctoral work at the University of Florida under the supervision of Dr. Dan Stenzen and then Dr. David E. Richardson. While at the University of Florida, she received the Guffey teaching award and the president's outstanding student award twice.

Cyndy married Jacob S. Buegers, a fellow chemistry doctoral candidate, at London Grove Friends' Meeting house in London Grove, Pennsylvania on July 23, 1988. Upon completion of her doctoral work, she will begin a postdoctoral research position in the chemistry department at Rutgers University in New Brunswick, New Jersey under the supervision of Dr. Stephen Leick.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


David B. Richardson, Chairman
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Sam Brown, Jr.
Associate Professor of Food Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


George H. Nycholatich
Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


Earl Stouffer
Associate Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.


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This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1988

Dean, Graduate School